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POSSIBILITY OF DIRECT TRANSPORTATION OF TESTICULAR HORMONE TO SOME TARGET ORGANS LOCATED CLOSE TO THE TESTICLE IN THE POSTNATAL RAT AND ITS PHYSIOLOGIC SIGNIFICANCE

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THE seminal vesicles and prostates of rats are highly sensitive to androgen. In the hypophysectomized animal the recovery and growth of the regressing organs can be easily obtained by the administration of minute doses of androgen. The maintenance (Cutuly *et al.*, 1938) or reinitiation (Nelson, 1940) of spermatogenesis and the scrotal growth, however, were not accomplished, unless animals were treated with much larger amounts of androgen.

In the study of the course of sexual maturation some paradoxical facts came to attract our attention. In our colony of rats, the significant growth of the accessory sexual organs (seminal vesicles and prostates) was never observed until about 60 days of age, while spermiogenesis, descent of testis and epididymal growth began to take place almost simultaneously at the 30–40th day of age (Suzuki, 1950a,b, 1951, 1952a,b, 1954a,b.).

Why do the proper accessories fail to start their significant growth at this earlier stage of the maturation process, though these organs, as Price *et al.* (1944) have reported, easily react with exogenous androgen even before the 26th day of age?

Dvoskin (1943, 1944) obtained the local maintenance of spermatogenesis in hypophysectomized rats by the intratesticular implantation of a pellet which contained an amount of testosterone much less than necessary for complete maintenance of the accessory organs, and clearly proved that the gametogenetic stimulation of androgen was here a direct action. He also

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suggested that the local action of the hormone on spermiogenesis may be a physiologic process. On the other hand, Jost and some workers (cf. Jost, 1953) have reported that in the foetal life, testicular secretion migrates and directly stimulates the development of the male sexual accessories and suppresses that of female ones, though this secretion seems to be slightly different from the testis hormone in the adult life.

From the study of the epididymal growth under several experimental conditions in the rats, the present authors obtained a concept that the testis hormone is transported not only by the systemic blood circulation but also by a direct route to the targets adjacent to the testicle even in the postnatal life, and this may furnish a favourable explanation for the present paradox.

MATERIAL AND METHODS

As experimental animals, male rats of the Wistar strain reared in our laboratory were used.

The outline of the plan and procedures of the experiments is given in Figure 1.

Experiment 1

Operations were performed at the 25th day of age and the rats were sacrificed at various intervals after the operation. In all groups the left testis was removed.

Group 1. The right testis remained intact.

Group 2. The efferent ductules of the right testis ligated.

Group 3. Bilaterally orchiectomized; control of Groups 1 and 2.

Group 4. Ligations applied on the testicular branch of internal spermatic blood vessels and on the efferent ductules at the right side.

Group 5. Bilaterally orchiectomized; control of Group 4.

Experiment 2

Rats 20 or 25 days old were used and sacrificed four days after the operation. The left testis remained intact. All animals received a single injection of Penicillin in oil (15,000 I.U.) immediately after the operation.

Group 6. The right testes implanted with following materials.

a) Anterior pituitaries of adult male rats about 200 days old were pooled and desiccated in acetone, used as minute pieces or a small pellet (dose: from 0.1 to 6.0 mg.).

b) Inactivated pituitary material treated at 100° C. for 4 hours (dose: 0.6 mg.).

c) Sterilized methyltestosterone-cholesterol pellets incorporated with 1.0, 2.5, 5.0 and 25.0% of the hormone (weight of pellet: 1.0–1.5 mg.).

d) Sterilized cholesterol pellet (weight of pellet: 1.0–1.5 mg.).

e) Acetone-desiccated pieces of cerebral cortex, liver and kidney from adult male rats (dose: 0.6 mg.).

Implants were inserted into the caudal end of the right testis, just underneath the tunica albuginea, with the aid of a glass capillary syringe.

Group 7. Sham operated at the right testis.

Experiment 3

Operations were performed at various days of age and sacrificed four days after the operation.

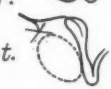
EXPT. NO.	GROUP NO.	NOS. OF RATS	RIGHT SIDE LEFT SIDE	AGE AT OPER. & AUTOPSY
1	1	22	Intact Orchiect.	Operated at 25th day
	2	26	Lig.(X) of Effer. d. "	Autopsied at Various days after the Oper.
	4	26	Lig.(X) of Effer.d. + Int.Sper. "	
	3	33	Orchiect.  "	
	5	24		
2	6	105	Implt. Pituitary etc. Intact	Operated at 20 or 25th day Autopsied at 4 days after
	7	26	Sham Oper. "	
3	8	130	Intact Orchiect.	Operated at Various days of age Autopsied at 4 days after
	9	33	" Lig.(X) of Effer. d.	

FIG. 1. Plan and procedures of the experiments. N.B. 1, testis. 2, epididymis. 3, deferent duct. 4, efferent ductules. 5, testicular branch and 6, epididymal branch of internal spermatic blood vessels. 7, deferential blood vessels.

Group 8. The left testicle removed, the right intact.

Group 9. The left efferent ductules ligated, the right intact.

At autopsy, epididymides and deferent ducts were weighed to the nearest tenth mg. and the other reproductive organs to the nearest mg., and as an index of the growth of the proper accessory sexual organs the weight of the seminal vesicles including the coagulating glands (combined both sides) were used. Smears of the testicular and epididymal contents were examined for the first appearance of the sperm.

In some representative cases, the tail of epididymis was examined histologically. The materials were fixed in Bouin's and in Zenker-formol fluids. For the staining of general histological purpose Heidenhain iron-hematoxyline & light-green and Heidenhain's azan stain, for connective tissue Lillie's allochrom stain (Lillie, 1951) and for tissue basophilia eosin-methylene blue (citric acid-disodium phosphate buffer, pH 4.2–5.0 and RNA staining was controled by ribonuclease digestion) were used. Small pieces fixed in chilled 80% alcohol were used for the Gomori's procedure in alkaline phosphatase detection (Gomori, 1952), serving Na-glycerophosphate as a substrate.

The weight of the reproductive organs were expressed in the comparative weight of the test organ to body weight (mg. organ weight per gm. body weight), and difference in the growth of the epididymides or of the deferent ducts of one individual were expressed by the right-left weight ratio (asymmetric ratio).

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RESULTS

Experiment 1. Asymmetric growth of epididymides in unilaterally orchiectomized rats

The growth of the separated right epididymis in Group 2 was inferior to that of the intact one in Group 1. The difference, however, was not significant until 60 days of age, when the sperm began to be detected in the caudal part of the intact epididymis. On the other hand, a significant growth difference was found between the separated epididymis with testis and the epididymis without testis in Group 2. Thus their asymmetric growth was clearly established (Fig. 2).

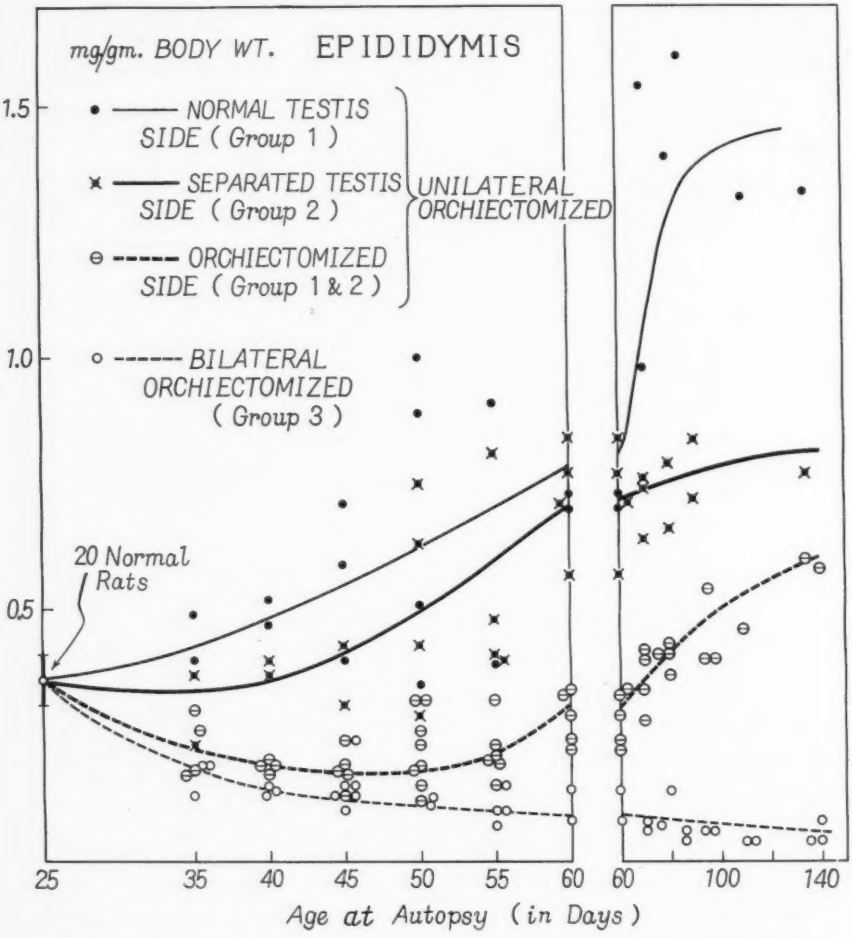

FIG. 2. Epididymal growth curves of rats under several conditions (Exper. 1). Animals were operated at 25 days of age.

The size of the left epididymis without testis and the seminal vesicles remained the same as those of the bilaterally orchiectomized controls until 50 days of age, and thereafter these organs grew steadily (Fig. 2 and Fig. 3).

In Group 4, in addition of the ligation of the efferent ductules, the blood supply to the separated testis was intercepted by ligating the testicular branch of internal spermatic. The testis thus operated underwent almost complete degeneration following the operation. At 10, 15, 20 and 25 days after the ligation they weighed respectively 70, 48, 39 and 42% as much as the removed testes.

The epididymis and deferent duct of orchiectomized side and the seminal vesicles took the same course of atrophy as in the bilaterally orchiectomized controls (Group 5) during the course of the experiment (Fig. 4). On the other hand, the separated epididymis and deferent duct of the right side, located at the ligated testis, continued further growth for a certain length of time.

This may indicate that a certain substance (maybe androgen) which was contained in the ligated testis at the time of operation exerted its local androgenic action effectively upon the adjacent epididymis and deferent duct.

Experiment 2. Does the inequality of androgen production or of androgen content in the testes between the right and left sides cause the asymmetric growth of epididymides and deferent ducts?

Following the implantation of pituitary material, the bilateral testes in-

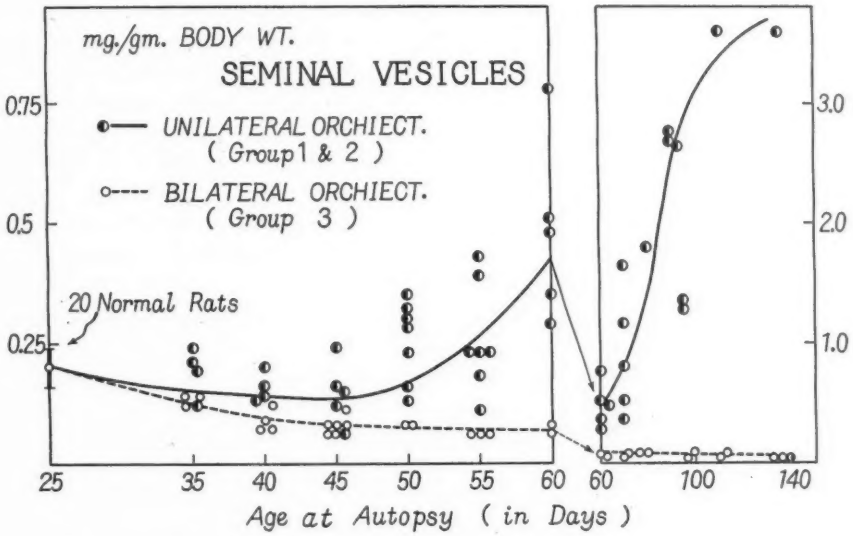

FIG. 3. Growth of seminal vesicles in uni- and bilaterally orchiectomized rats (Exper. 1). Animals were operated at 25 days of age.

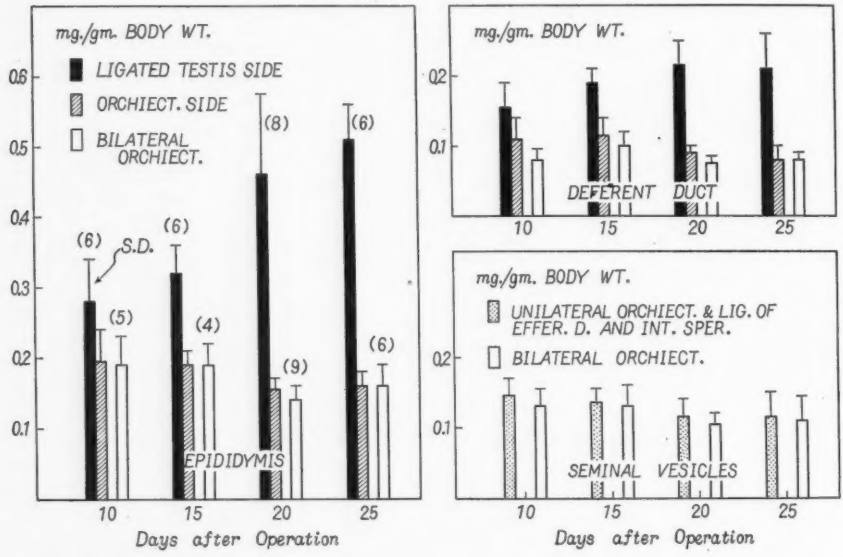

FIG. 4. Asymmetric growth of epididymides and deferent ducts in unilaterally orchiectomized rats whose remaining testis was intercepted from blood supply and separated from the related epididymis (Group 4 in Exper. 1). The littermates were bilaterally orchiectomized as for the control (Group 5 in Exper. 1). Animals were operated at 25 days of age. Number in parentheses indicates the numbers of rats. Range of distribution is expressed in standard deviation (S.D.).

creased in weight, and the highest increase was attained as much as 30% over their littermate controls with increasing doses of implants, but the right implanted one was always slightly heavier than the contralateral one. In the cases of androgen implants, the growth of testis was suppressed bilaterally comparing their littermate controls which received cholesterol, but the right implanted testis were less affected than the left intact. In the sham operated animals and in the animals implanted with non-pituitary materials, the treated testicle was a little lighter than the left intact, especially in the animal operated at 25 days of age.

Classified by the sorts of materials, the right-left weight ratio (asymmetric ratio) of epididymides and that of deferent ducts are shown in Figure 5A, and also in the epididymis and the deferent duct of the non-implanted testis side and in the seminal vesicles, their percentage weight increase over those of the littermate controls are given in Figure 5B.

The asymmetry of growth induced by implantations of anterior pituitary and methyltestosterone was not present in any of the controls (Group 6 b, d, e and Group 7). The epididymides and the deferent ducts of the implanted testes with doses more than 0.4 mg. of the dry pituitary and with the androgen implants, became decidedly heavier than those of the

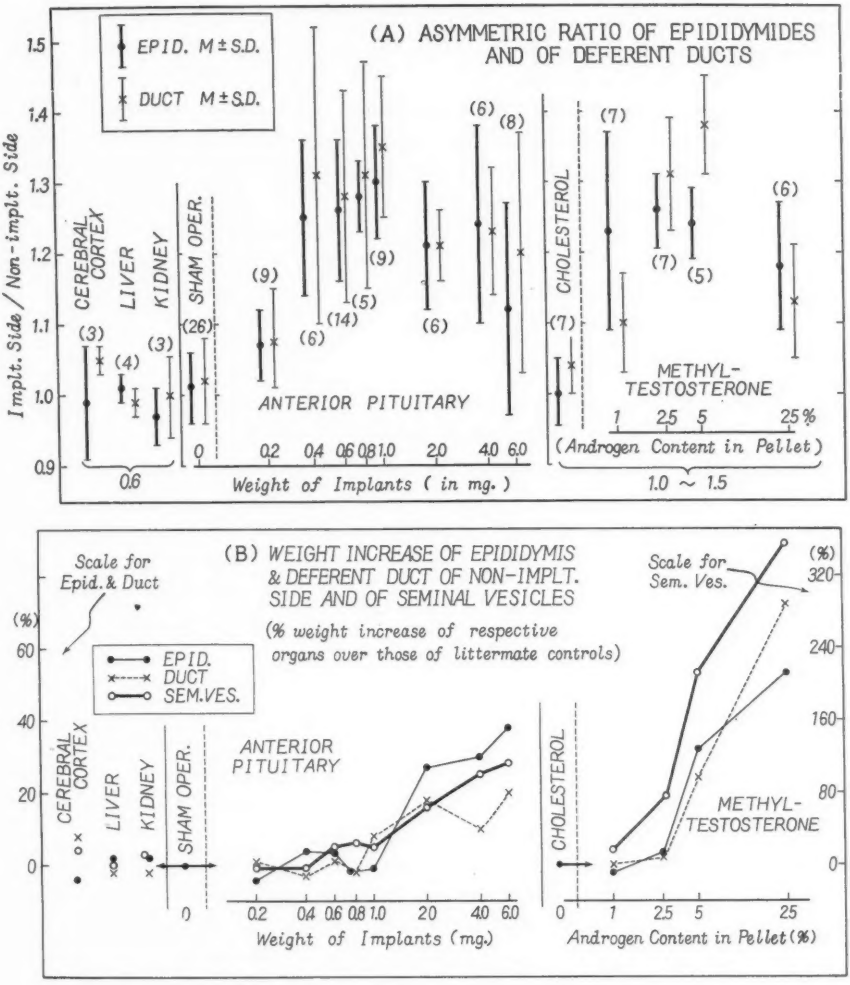

FIG. 5 A. and B. Unilateral intratesticular implantation of desiccated anterior pituitary or methyltestosterone pellet, etc. (Exper. 2). Animals were implanted or sham operated at 20 or 25 days of age and sacrificed four days after the operation. Range of distribution is expressed in standard deviation (S.D.). Number in parentheses indicates numbers of rats.

contralateral ones. No parallelism, however, was observed between the asymmetric ratio of these organs and either the doses of pituitary implants or the concentrations of androgen, because by increasing of the amounts of them, the organs of the left non-implanted testis side were also stimulated and increased their weight as shown in Figure 5B. Consequently the asymmetric ratio became less significant in the implantations of doses more than

2.0 mg. of the pituitary and of 25% androgen pellet, while on the contrary, weight increase of the seminal vesicles, stimulated by the blood circulating androgen, became evident.

Besides these implantations, experiments with the fresh anterior pituitary were made as in the same condition stated above. A significant asymmetry in the epididymides and in the deferent ducts was obtained by implantation of from a half to one and a half of anterior pituitaries of normal adult male rats. But in the cases where much larger numbers of the glands or a gland derived from castrated male were implanted, the asymmetric ratio became far less significant, and simultaneously the stimulation on the seminal vesicles and on the epididymis and deferent duct of the non-implanted testis became evident.

Experiment 3. Physiologic significance of the direct transportation of testis hormone on the growth of epididymis and deferent duct.

The growth ratio of the right and left epididymides and of the deferent ducts in the unilaterally left orchiectomized rat (Group 8) and in the case of the animal with an intact right testis and the left one with a ligation on its efferent ductules (Group 9) were followed during the early stage of the sexual maturation. The results are shown in Figure 6. (The results with the deferent ducts are excluded because they were almost the same as those of the epididymides.)

In Group 9 the asymmetric growth was not detected. In Group 8, the asymmetry due to the direct action of testicular substance and its absence on the orchiectomized side, however, was significant and the asymmetric growth ratio was highest at about 30 days of age. After about 50 days of age, the ratio of asymmetry started to decline, though the weight of their seminal vesicles began and continued to increase.

The slight asymmetry also was observed among some animals in Groups 8 and 9, operated after 50 days of age, when sperm began to be detected in the tail of the right intact epididymis. This asymmetry should be attributed to the additional quantity of sperm transferred into the right epididymis, because the quantity of sperm found in the head of the right and left epididymides at autopsy differed widely.

HISTOLOGICAL NOTE

In groups of the unilateral orchiectomy with ligation of efferent ductules (Group 2), and of the unilateral intratesticular implantations of pituitary materials and of the androgen (Group 6 a and c), there was no essential histological difference between the right and left epididymides by the mitotic activity, the staining reactions, the site of alkaline phosphatase activity and the relative height of epithelium, despite a significant asymmetry in the size of epididymides having been detected in these cases. That is, the difference in epididymides was detected in quantitative but not in

the qualitative nature. None of the epididymides showed any inflammatory change and no abnormal proliferation of connective tissue suggestive of non-specific growth.

By the implantations of either pituitary materials or androgen pellets, essentially the same histological appearance was obtained. The growth of all elements of the epididymides of the two sides, preserving their normal

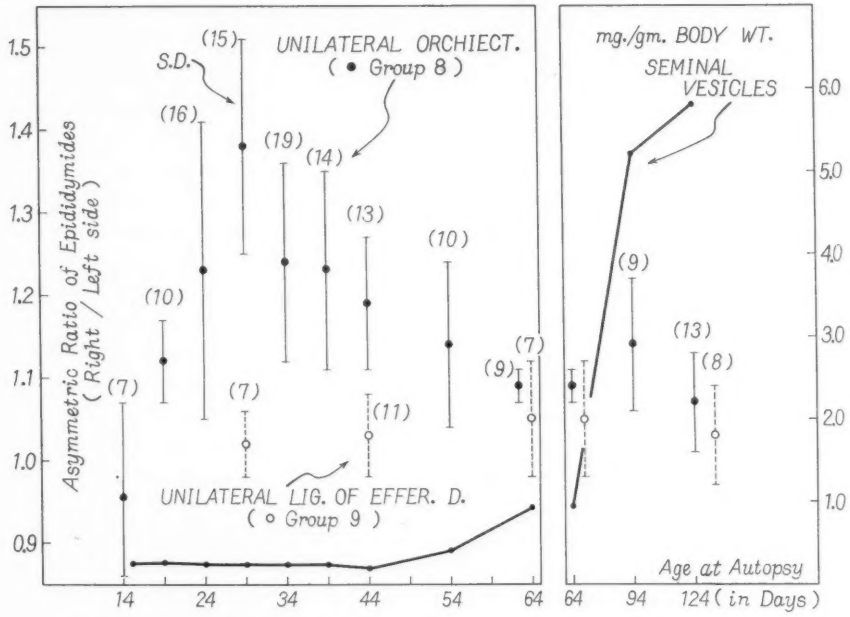

FIG. 6. Efficacy of local androgenic action of the testicular substance on the epididymal growth during maturation process (Exper. 3). Animals were operated at various days of age and sacrificed four days after the operation. Number in parentheses indicates numbers of rats. Range of distribution is expressed in standard deviation (S.D.).

pattern, predominated considerably over those of the littermate controls (sham operated and cholesterol implanted), especially in number of mitoses in parenchyma and in stroma cells.

On the other hand, the epididymides of bilaterally orchiectomized rats (Group 3 and 5) could be distinguished histologically from those of the above mentioned groups (Groups 2, 6 and 7) but even in these cases the difference was not so striking. In this connection, Cieslack (1944) has already pointed out that the androgenic effect on the epididymis can be more easily detected by weight increase than by histological change.

The histological changes were not adequate to visualize the local action of testicular androgen on the epididymis, however, it is to be noted that in the conditions used in the present study, the epididymides of the orchi-

ectomized side and those of the non-implanted side could respond to a minute amount of androgen in the general circulation.

DISCUSSION AND CONCLUSIONS

The findings obtained in the present study may present an evidence of local androgenic action of a certain testicular substance (may be androgen) upon the epididymis and deferent duct in the physiologic state as well as in the experimental conditions.

This local action manifests a physiologic significance only when the level of androgen in the circulating blood is in the critical or threshold range for the induction of the growth of seminal vesicles as can be seen in Figure 6 (Group 8), where the asymmetries of epididymides or of deferent ducts are highest at around 30 days of age when the epididymal growth becomes significant in the physiologic sexual maturation process (Suzuki, 1954a, b), and thereafter these start to decline with the progressive increase of the weight of seminal vesicles. This decline of the asymmetric ratio is not interpreted to mean the decreasing in the local action, but the asymmetries of these organs are overshadowed by the systemic effect of androgen in the circulating blood which results in the weight increase of epididymis and deferent duct of the orchiectomized side. Because, the same relation between the asymmetric ratio and the response on the seminal vesicles was obtained not only in the case of implantation of pituitary materials but also in the case of androgen implantation as can be seen in Figure 5A and B.

This parallelism and the finding of Cieslack (1944) who has demonstrated the testicular secretion of androgen as early as the 8th day of age in the rat, using the weight increase of the tail of epididymis as the criterion, may indicate that the testicular substance which is directly transported to the adjacent targets in the early stage of the postnatal life (around 30 days of age) should be the same with natural androgen secreted by the testis in the stage of adult life. For the conclusive evidence to identify this substance, further studies are necessary.

From the results in Experiment 1 (Groups 2 and 4), the local action is possibly caused by diffusion of androgen through the tunica albuginea into the adjacent targets, because there is no evidence for the presence of special blood communication between the separated testis and its epididymis and deferent duct, and the efferent ductules were completeiy ligated. In the physiologic state, it is probable that the androgen can be also carried by the testicular secretion through the efferent ductules into the epididymis, as Tuovinen *et al.* (1947) have suggested. The local effect by this route, however, seems here to be less important as can be seen in Figure 6 (Group 9) where the growth of the normal epididymis and another one at the separated testis is compared.

By the concept of direct transportation of testicular androgen, the paradox observed in the sexual maturation process may be reconciled as follows: in 30–50 days of age, quantity of androgen in the circulating blood is not quite enough yet to induce a significant growth of seminal vesicles, even though the androgenic effects could be demonstrated in the epithelium of this gland by histological examination. (The halo-like granules of the epithelial cells are observed at about 35 days of age.) On the other hand, epididymis, deferent duct and scrotum which receive additional androgen from the direct route are able to start their significant growth. In other words, between two kinds of target organs, namely one adjacent to the testis and the other remote, there exists a considerable quantitative difference in fixation or utilization of androgen in this stage of life.

SUMMARY

The paradox, observed in the sexual maturation process in the rat that the epididymal growth, descent of testis and spermiogenesis occur at about 30–40 days of age, much earlier than the significant growth of seminal vesicles and prostates, may be reconciled mostly by a concept in which the testis hormone is transported not only by the systemic blood circulation but also by a direct route to the targets adjacent to the testicle. Between two kinds of targets, one adjacent to the testis and the other remote, therefore, there exists a quantitative difference in fixation and utilization of androgen in this early postnatal live.

The results which support this concept are as follows: 1) By the unilateral orchiectomy in immature rats, the growth of separated epididymis at the testis side predominated over that of the contralateral orchiectomized. 2) The influence of the epididymal content of testicular secretion upon the weight increase of epididymis was not significant before 60 days of age when sperm began to be detected in the tail of intact epididymis. 3) The conditions of inequality of the androgen content or of the androgen production between both testes, provided by the unilateral intratesticular implantations of androgen pellets or of anterior pituitary materials in immature animals, induced a significant asymmetric growth of the epididymides and deferent ducts. By the overdosage of the pituitary and of the androgen, the asymmetry became less significant, due to the weight increase of these organs of non-implanted testis side, and simultaneously the stimulating effect on seminal vesicles became evident. 4) The growth asymmetry caused by unilateral orchiectomy of four days period was followed during the course of sexual maturation. The ratio of asymmetry was found to be the highest at about 30 days of age, before any significant growth of seminal vesicles had occurred. Thereafter this ratio started to decline, though the weight of their seminal vesicles began and continued to increase.

REFERENCES

CIESLACK, E. S.: *Endocrinology* **35**: 63. 1944.

CUTULY, E., E. C. CUTULY AND R. D. MCCULLAUGH: *Proc. Soc. Exper. Biol. and Med.* **38**: 818. 1938.

DVOSKIN, S.: *Proc. Soc. Exper. Biol. and Med.* **54**: 111. 1943.

DVOSKIN, S.: *Am. J. Anat.* **75**: 289. 1944.

GOMORI, G.: *Microscopic Histochemistry, Principles and Practice.* University of Chicago press, p. 180. 1952.

JOST, A.: *Recent Prog. in Hormone Res.* **8**: 379. 1953.

LILLIE, R. D.: *Am. J. Clin. Path.* **21**: 484. 1951.

NELSON, W. O.: *Am. J. Physiol.* 129 Proceedings: 430. 1940.

PRICE, D. AND E. ORITZ: *Endocrinology* **34**: 215. 1944.

SUZUKI, Y.: *Med. and Biol.* (*J*) **17**: 98. 1950a.

SUZUKI, Y.: *Med. and Biol.* (*J*) **17**: 156. 1950b.

SUZUKI, Y.: *Med. and Biol.* (*J*) **18**: 199. 1951.

SUZUKI, Y.: *Jap. J. Vet. Sci.* **14**: 116. 1952a.

SUZUKI, Y.: *Report of II. International Congress of Physiology and Pathology of Animal Reproduction and of Artificial Insemination.*: Vol. 1, 53. Copenhagen. 1952b.

SUZUKI, Y.: *Jap. J. Vet. Sci.* **16**: 1. 1954a.

SUZUKI, Y.: *Jap. J. Vet. Sci.* **16**: 87. 1954b.

TUOVINEN, P. I. AND R. POHJOLA: *Acta Endoc.* **3**: 1. 1949.

RAT PLACENTAL HORMONAL ACTIVITIES CORRESPONDING TO THOSE OF PITUITARY MAMMOTROPIN

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MANY earlier reports, now too numerous to list in any but a review paper, have emphasized the importance of the placenta as an endocrine organ. In the past 30 years this emphasis has been placed largely on such of its hormones as directly or indirectly influence the organs of reproduction, and therefore come to bear on the form and function of the placenta itself. In studies on the hormonal factors concerned with the maintenance of pregnancy few findings have been so striking as those showing that the rat placenta is capable of carrying out roles usually assigned to the pituitary. These roles entail the responsibility, not only of setting up the conceptus through the influence of gonadotropic secretions on the gonads, but also of maintaining it through trophic influences of carefully balanced proportions of these same hormones on the ovaries, and in turn the trophic influences of the gonadal hormones in proper ratios on the uterus and eventually the conceptus.

Since the mammary apparatus develops step by step with the conceptus, it seemed likely that some or all of the hormones influencing one might also be acting upon the other. In an earlier publication, data on the placental factors concerned with the maintenance of pregnancy in the rat have been presented (Averill *et al.*, 1950). These confirmed the earlier work of Astwood and Greep (1938, 1939) showing that a rat placental substance, "cyonin," stimulates the corpus luteum to secrete progestin. Since this luteotropic activity is only one of the roles attributed to the purified pituitary hormone, mammotropin (prolactin), it seemed important to make further comparisons of the placental and pituitary factors.

METHODS

In the following experiments the female sex hormones were injected in the form of estrone and progesterone. In referring to unidentified substances with estrogenic or progestogenic activities the terms estrin or progestin respectively are used. The term interstitial cell stimulating hormone (ICSH) is used as synonomous with luteinizing hormone (LH). Since the follicle stimulating hormone (FSH) is necessary in combination with ICSH as a stimulant to ovarian production of estrin, such an effect on the ovary is

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referred to as an ICSH-FSH effect. The term mammotropin (MH) is used to designate the hormone of the anterior pituitary, which in its purest form has: (*a*) a crop stimulating effect in pigeons; (*b*) a luteotropic (not luteinizing) effect, and this term refers to the hormone's power of causing the production and secretion of progestin by ovarian luteal tissue and thus indirectly acting upon the mammary gland to induce lobulo-alveolar growth; (*c*) a direct mammary growth stimulating (mammotropic) effect obtained in synergism with estrin and progestin; (*d*) a lactogenic or galactopoietic effect. Although other effects have been ascribed to this hormone, they are of no concern here. Our purpose, then, was to show that activities corresponding to these four effects of purified pituitary mammotropin, reside for a time in the mid-pregnancy rat placenta.

The test used for detecting crop sac stimulation was the "local" method introduced by Lyons and Page (1935). This is a modification of the original assay devised by Riddle *et al.* (1932) in pigeons and doves.

One of the earliest demonstrations of a luteotropic effect of MH was that of Dresel (1935) who showed what may now be interpreted as a prolonged luteal phase in mice injected with a commercial prolactin. This estrus-inhibiting effect was studied in this study of placental activities.

The test for a mammotropic effect was one formerly used by Lyons (1943). Hypophysectomized, oöphorectomized virgin rats do not show mammary growth following injections of estrone, progesterone or a combination of these hormones. If MH be injected simultaneously with estrone and progesterone, lobulo-alveolar growth may be induced. STH added to the above 3 hormones causes a more complete lobulo-alveolar response in such animals (Lyons *et al.*, 1952). Some rats were hypophysectomized only and then injected with estrone and rat placentae to demonstrate simultaneously the luteotropic and mammotropic activities. In these experiments the intention was to induce the rat ovary to form progestin. This hormone would be expected to synergize with the injected estrone and the rat placenta to cause lobulo-alveolar mammary growth.

Several tests were available for lactogenic or galactopoietic activity of the rat placentae. Nelson and Gaunt (1936) using hypophysectomized guinea pigs demonstrated the importance of adrenocortical as well as pituitary activity in galactopoiesis. Lyons and Pencharz (1938) induced milk secretion in hypophysectomized rats with crude MH containing ACTH and STH. More recently (Lyons *et al.*, 1952, 1953b) have shown the need for 4 hormones (estrin, progestin, MH & STH) in the induction of complete lobulo-alveolar growth, and of 3 hormones (MH, STH plus either cortisone or ACTH) in establishing milk secretion in such glands. The animal used was the hypophysectomized, oöphorectomized virgin rat. In testing rat placentae for lactogenic potency this same form was used in the present experiment.

In the final experiment to be described different parts of the rat placentae were tested for mammotropic activity using the method mentioned above.

EXPERIMENTAL

Experiment 1.—The Crop Sac Stimulating Effect of Rat Placenta

Saline extracts of 12-day placentae were injected daily for 4 days, locally between the skin and the crop sacs of 1-month-old Silver King squabs. Total dose equivalents, ranging from 0.03 to 4.0 placentae were administered to 8 groups of 3 birds. The tests were read on the day following the last injection.

Doses of 0.03, 0.06, 0.12 and 0.5 placentae gave negative responses. Total doses of 0.25 and 1.0 placentae, each elicited a border-line reaction in 1 of 3 birds. Positive reactions were obtained in all birds injected with either 2 or 4 placentae.

Experiment 2.—The Luteotropic Effect of Rat Placenta

Daily doses of 1 to 3 placental equivalents were administered to 20 normal rats in which the vaginal cycle was followed by the smear technic. The rats were injected for 10 days and the test was considered positive if vaginal cornification was prevented for 10 days. The first inguinal mammary gland was dissected out and prepared as a whole mount by a technique already described (Lyons and Johnson, 1953a). The state of mammary development was assessed by 7-power microscopy and expressed according to an arbitrary scale. A "D" rating was given to a mammary gland showing a duct system either completely devoid of alveolar buds, or else containing a negligible number of these (Fig. 3). "DAB" referred to a gland with sparsely distributed alveolar buds on a well developed duct system (Fig. 2). The rating "LA" was assigned to a gland with lobulo-alveolar development (Fig. 1). An arbitrary rating from "LA 1" to "LA 4" was assigned depending upon the size and number of lobules present. Since the degree of development varied in different areas of the same gland, the rating given represented an average assessment of the gland and in some instances involved fractions as well as whole numbers.

Observations on the mammary glands and estrous cycles of the rats are shown in Table 1. In 3 rats injected with three 12-day placentae daily, (group 4), the vaginal cycle was stopped in a prolonged mucification phase; and the mammary glands showed excellent lobulo-alveolar development (Fig. 1). Daily doses of 1 or 2 placentae inhibited the cycle in 7 of 10 cases; and caused L-A mammary growth in 8 of the rats (groups 1–3). Two of the three rats in which the cycle was not inhibited showed L-A growth. Only 1 of the 7 rats in which the cycle was inhibited failed to show L-A growth.

TABLE 1. OBSERVATIONS ON THE MAMMARY GLANDS AND ESTROUS CYCLES OF NORMAL RATS INJECTED WITH PLACENTAL PREPARATIONS FOR TEN DAYS

Group	Rats	Placentae injected per day	Estrous cycle inhibited	Mammary gland
1	3	1 12-day	no no yes	DAB (1) LA2 (1) LA4 (1)
2	3	2 12-day	no yes yes	LA1 (1) LA3 (1) LA4 (1)
3	4	2 12-day*	yes yes yes yes	DAB (1) LA1 (1) LA2 (1) LA3 (1)
4	3	3 12-day	yes	LA4 (3)
5	3	1 15-day	no yes	DAB (2) DAB (1)
6	3	3 15-day	no	DAB (3)
7	1	1 17-day	no	DAB (1)

Abbreviations: DAB = Ducts and alveolar buds.
LA = Lobulo-alveolar, 1+ to 4+.
* A 2% butanol extract; other preparations were saline suspensions.
In parentheses: the number of rats showing the mammary reaction indicated

The administration of 15 or 17 day placentae stopped the cycle in only 1 case, and failed to induce L-A development (groups 5–7). The ovaries of the animals in which the cycle was inhibited showed maintained corpora lutea with the cell hypertrophy typical of luteotropic stimulation.

Experiment 3.—The Mammotropic Effect of Rat Placenta

Long-Evans virgin rats, 2–7 months old were hypophysectomized and oöphorectomized; and injections of estrone, progesterone and placental preparations were begun on the same day. The placentae administered were taken from normal rats on the 8th, 10th, 12th, 13th, 15th, and 17th days of gestation, counting day 1 as the day of finding sperm in the vaginal smear. Average weights of such placentae were 7, 34, 55, 100, 209, and 307 mgm. respectively. In the case of placentae from the 10th day of gestation and later it was possible to eliminate the fetuses and their amniotic sacs and administer only the combined fetal and/or maternal parts of the placentae. The specimens from the 8th day of gestation were so small that the entire conceptus was administered. Placentae were administered subcutaneously in the form of fresh tissue implants, or as saline extracts or suspensions in daily doses ranging from 0.5 to 11 placental equivalents per day. Injections were begun immediately after the operations and continued for varying lengths of time. The ovarian hormones estrin and progestin were given in subcutaneous injections of 0.2 cc. sesame oil containing either 1 μg. of estrone, 4 mg. of progesterone, or both. Necropsy was performed on the day following the last injection and mammary glands were graded as in Experiment 2.

The effects of the hormonal injections on the mammary glands are coded in Table 2. The body weight change is also recorded. The loss in weight of untreated rats during the first 10 days following the double operation usually ranged between 1 and 2 gm. per day. There seemed to be a tendency for the rats injected with the younger placentae to lose less weight than those of the other groups and the controls; but this is considered meager evidence for a placental growth hormone.

During the 10 post-operative days the mammary glands of the rats treated with sesame oil (the vehicle for estrone and progesterone) regressed to a bare duct system (Fig. 3). This regression was not prevented by daily injections of 1 μg. of estrone and 4 mg. of progesterone. Some of the rats in all of the groups injected with placentae as well as estrone and progesterone showed lobulo-alveolar growth. Others showed maintenance of alveolar buds (DAB), an effect obtainable in such animals with purified MH alone (Fig. 4). Eleven 8-day placentae (77 mg.) daily induced L-A growth in 3 of 5 tests. The 12-day placentae were consistently potent in the mammotropic factor. A single placenta implanted daily induced L-A growth in all of 5 rats treated for only 6 days and in all of 7 rats treated for 10 days. Using a saline extract of 12-day placentae, postive reactions were obtained in 2 of 4 rats at a dose equivalent to half of a placenta. Some of the rats in the groups injected with the 13, 15, and 17-day placentae showed L-A growth, but the response was less in proportion to the weight of placentae injected than that obtained with the 12-day placentae.

TABLE 2. EFFECTS OF INJECTIONS OF OVARIAN AND PLACENTAL PREPARATIONS ON THE MAMMARY GLANDS OF HYPOPHYSECTOMIZED-OÖPHORECTOMIZED RATS

Group	Rats	Placentae per day	Ovarian hormones daily dose	No. days injected	Body wt. change	Mammary glands
1	10	None	Sesame oil	6	−25 gm.	D
2	10	None	1 μg.E+4 mg. P	6	−30 gm.	D
	10	None	1 μg.E+4 mg. P	10	−30 gm.	D
3	5	11 8-day FT	1 μg.E+4 mg. P	10	−14 mg.	DAB (2) LA1 (1) LA2 (2)
4	5	2 10-day FT	1 μg.E+4 mg. P	10	−13 gm.	DAB (4) LA1 (1)
5	5	1 12-day FT	1 μg.E+4 mg. P	6	−16 gm.	LA1
	7	1 12-day FT	1 μg.E+4 mg. P	10	−19 gm.	LA1 (6) LA2 (1)
	1	2 12-day FT	1 μg.E+4 mg. P	10	−26 gm.	LA2
	3	3 12-day FT	1 μg.E+4 mg. P	6	−16 gm.	LA1
	2	3 12-day FT	1 μg.E+4 mg. P	10	−10 gm.	LA2
	1	5 12-day FT	1 μg.E+4 mg. P	10	−13 gm.	LA3
	1	7 12-day FT	1 μg.E+4 mg. P	10	− 6 gm.	LA2
	1	9 12-day FT	1 μg.E+4 mg. P	10	− 2 gm.	LA2
6	4	0.5 12-day SE	1 μg.E+4 mg. P	10	−14 gm.	DAB (2) LA1 (2)
	4	1 12-day SE	1 μg.E+4 mg. P	10	−15 gm.	LA1
	4	1.5 12-day SE	1 μg.E+4 mg. P	10	− 9 gm.	DAB (1) LA1 (3)
	4	2 12-day SE	1 μg.E+4 mg. P	10	−13 gm.	DAB (1) LA1 (2) LA2 (1)
	4	3 12-day SE	1 μg.E+4 mg. P	10	−15 gm.	LA2
	4	4 12-day SE	1 μg.E+4 mg. P	10	−14 gm.	LA2
	4	6 12-day SE	1 μg.E+4 mg. P	10	−17 gm.	LA2 (3) LA3 (1)
7	1	2 13-day SE	1 μg.E+4 mg. P	10	−25 mg.	LA1
	4	3 13-day SE	1 μg.E+4 mg. P	10	−20 gm.	DAB (1) LA1 (3)
8	4	1 15-day FT	1 μg.E+4 mg. P	6	−10 gm.	DAB
	1	1 15-day FT	1 μg.E+4 mg. P	10	−26 gm.	LA1
	1	3 15-day FT	1 μg.E+4 mg. P	10	−22 gm.	LA2
9	1	1 17-day FT	1 μg.E+4 mg. P	10	−32 gm.	DAB
	1	3 17-day FT	1 μg.E+4 mg. P	10	−34 gm.	LA1
	1	5 17-day FT	1 μg.E+4 mg. P	10	−16 gm.	LA2

Abbreviations: D = ducts only; DAB = ducts and alveolar buds; LA1 to 4 = degrees of lobulo-alveolar development. Number of rats in parentheses only when reactions were variable within a group. FT = fresh tissue; SE = saline extract of placentae; E = estrone; P = progesterone.

Experiment 4.—Combined Luteotropic and Mammotropic Effects of Rat Placenta

Since the previous experiments had shown both luteotropic and mammotropic activity in the rat placenta an experiment was performed showing both of these activities in the same animal. The test animal was a mature, adult virgin rat hypophysectomized only, and injected for 6–10 days with placenta in some cases and placenta plus estrone

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TABLE 3. EFFECT OF INJECTIONS OF RAT PLACENTAE WITH AND WITHOUT ESTRONE ON THE MAMMARY GLANDS OF HYPOPHYSECTOMIZED FEMALE RATS

Rats	Placentae per day	Estrone	No. of days	Body wt. change	Mammary glands
4	1 12-day	1 μg.	6	−22	LA1
5	1 12-day	1 μg.	10	−28	LA1
2	2 12-day	1 μg.	10	−27	LA1
3	3 12-day	1 μg.	10	−22	LA1 (2) LA2 (1)
2	2 12-day	—	10	−18	DAB
4	3 12-day	—	10	−15	DAB (3) LA1 (1)
4	5 12-day	—	10	−12	DAB (1) LA1 (3)

Abbreviations as in Table 2.

in others. The intention was to stimulate the production of an equivalent of approximately 4 mg. of progestin (as used in the last experiment) by the rat's ovaries. If this induced L-A mammary growth similar to that obtained on the doubly-operated rats with estrone and progesterone and placentae, further support would be obtained for a dual function or nature of this hormone.

As may be seen in Table 3, all of the 14 rats injected for 6 or 10 days with 1 μg. of estrone and from one to three 12-day placentae showed L-A growth (Fig. 5). Of the 10 rats injected only with placentae, one rat at the 3 placentae and three at the 5 placentae daily levels showed 1+L-A growth. When these results were obtained, 3 hypophysectomized-oöphorectomized rats were injected with ten 12-day placentae daily for 10 days; and 2 of 3 showed L-A growth, one being 1+ and the other 3+. The average body weight loss was 13 gm.

Experiment 5.—Lactogenic Effects of Rat Placenta[1]

The fourth activity for which the rat placenta was tested in comparison with pituitary MH was that of milk secretion. For this purpose 8 hypophysectomized-oöphorectomized, adult virgin rats were injected with 1 μg. of estrone, 4 mg. of progesterone, 2 mg. of pituitary MH and 0.2 mg. of STH daily for one week. During the following week they were injected as follows: two with five 12-day placentae; 2 with five 12-day placentae plus 1 mg. hydrocortisone acetate (F); 2 with 1 mg. F alone; 2 with five 12-day placentae plus 1 mg. F plus 0.5 mg. STH. On the day after the last injection, the mammary glands were inspected for milk secretion; and spreads were made in the usual way for microscopic study.

In Table 4 may be seen the body weight change at the end of the injection period, and arbitrary assessments of the degree of mammary development and milk accumulation. The first week of treatment induced L-A

[1] Before developing a test for lactogenesis in the rat, resort had been made to the virgin guinea pig assay (Lyons, 1941). In 6 tests a total dose of sixteen 12-day placentae gave negative results in all; in 4 tests, 24 placentae were positive in 3; in 5 tests, 32 placentae were positive in 3.

TABLE 4. SHOWING DIFFERENT COMBINATIONS OF HORMONES THAT STIMULATE MAMMARY DEVELOPMENT AND LACTATION

Exper. 5	First phase					Second phase					
Rat	Estrone	Progest.	MH	STH	Plac.	Plac.	F	STH	Body wt. change	Mammary gland	Lactation
95	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	1 mg.	0.5 mg.	− 6	LA 2	2+
39	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	1 mg.	0.5 mg.	−13	LA 2	2+
74	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	1 mg.	—	−19	LA 1	1+
12	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	1 mg.	—	−11	LA 0.75	1+
64	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	—	—	+10	LA 0.75	0
42	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	—	—	+ 6	LA 0.25	0
08	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	—	—	−26	LA 0.25	0
86	1 μg.	4 mg.	2 mg.	0.2 mg.	—	5	—	—	−19	LA 0.25	0
Exper. 6											
65	1 μg.	4 mg.	—	—	4	6	—	—	−20	LA 1.5	0
81	1 μg.	4 mg.	—	—	4	6	—	—	−40	LA 2	1+
29	1 μg.	4 mg.	—	—	4	6	1 mg.	—	−30	LA 3	2+
71	1 μg.	4 mg.	—	—	4	6	1 mg.	—	−30	LA 3	2+
54	1 μg.	4 mg.	—	—	4	6	1 mg.	—	−29	LA 3.5	2+
98	1 μg.	4 mg.	—	—	4	6	1 mg.	0.5 mg.	−22	LA 3.5	3+
84	1 μg.	4 mg.	—	—	4	6	1 mg.	0.5 mg.	−19	LA 2.5	3+
97	1 μg.	4 mg.	—	—	4	6	1 mg.	0.5 mg.	−19	LA 3.5	3+

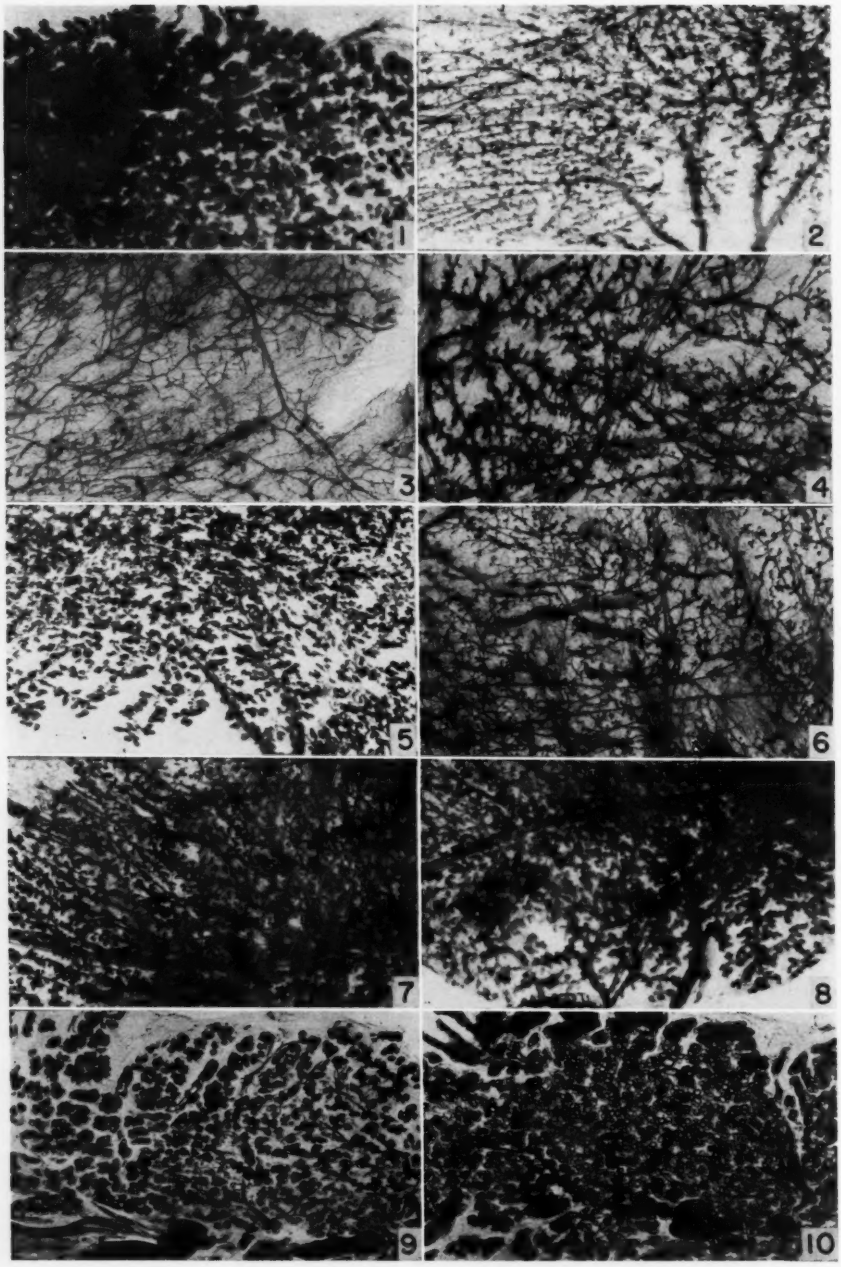
Note: In Experiment 5, phase 1 =7 days, and phase 2 =7 days.
In Experiment 6, phase 1 =6 days, and phase 2 =4 days.

growth in all of the rats. Those treated only with F during the second week showed mammary regression and no milk (Fig. 6). Placental extract plus F in the second week maintained the gland and induced beginning milk secretion. When STH was injected as well as these two substances the glands showed more distension with milk. Placental extract alone during the second week caused no gross lactation; but signs of beginning secretory activity were observed microscopically in the glands of one rat.

Experiment 6.—Combined Mammotropic and Lactogenic Effects of Rat Placenta

In this experiment 2% butanol extracts of the "fetal" part of 12-day rat placentae were used in both the developmental and secretory phases. After hypophysectomy and oöphorectomy the rats were prepared for 6 days with 1 μg. of estrone, 4 mg. of progesterone and 4 placental equivalents daily. Aside from controls used to show adequate stimulation after 6 days of treatment (Fig. 7), and untreated controls that have been repeated many times and shown to have atrophic glands, there were 8 rats in which the treatment was continued as follows for four days: 2 were injected with 6 placentae; 3 with 6 placentae plus 1 mg. of F; 3 with 6 placentae plus 1 mg. of F and 0.5 mg. of STH. Inspection and grading of the mammary glands was done as before.

The results are also listed in Table 4 and show that the 4 placentae synergized well with estrone and progesterone in inducing good L-A growth in 6 days; and that after discontinuing the injections of estrone and progesterone, further administration of the same placental extract induced beginning milk secretion in 4 days (Fig. 8). The rats injected with placenta plus F showed good milk secretion (Fig. 9); and those that also received STH showed the greatest engorgement with milk (Fig. 10). The



Mammary areas representing the first right inguinal gland. Spreads were fixed in formol, stained in alum carmine and embedded in plastic. Figures 9 and 10 are 0.1 mm.

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animals showed the usual post-operative weight loss, especially during the first 6 days. Thereafter only 2 (in the STH group) gained weight; the dose of 1 mg. of F being excessive, was only partially compensated for in the STH animals.

Experiment 7.—Assay of Different Regions of the Placenta for Mammotropic Activity

In a further attempt to localize the tissue concerned with the secretion or storage of the mammotropic activity, different regions of the 12-day rat placentae were isolated, stored in 2% butanol, homogenized and tested separately in the form of a suspension. The decidua basalis, as isolated, was found to be composed largely of glycogen laden decidual cells. Histologic sections of typical specimens also revealed a few penetrating trophoblastic elements including giant cells. This was due to imperfect separation from the Träger.

This otherwise discrete button of tissue weighs approximately 50 mg. at this stage of gestation; and was previously shown to have no more luteotropic activity than the combined Träger plus the decidua capsularis with associated yolk sac and trophoblastic cells which weigh 10–15 mg. The one region almost entirely maternal and the other fetal for the most part, were assayed separately for mammotropic activity, using adult hypophysectomized-oöphorectomized rats treated concurrently with 1 μg. of estrone and 4 mg. of progesterone. After 10 daily treatments the mammary glands were examined for evidence of lobulo-alveolar development.

A further step was taken when it was learned that the "fetal" placenta was more potent than the maternal. The "fetal" part was separated into: (1) The Träger plus

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celloidin sections. About $\frac{1}{3}$ of the gland is shown in most cases and magnified $4\frac{1}{2}\times$.

FIG. 1. From a normal rat injected daily with three 12-day placentae for 10 days. Lobulo-alveolar (L-A) development as in late pregnancy.

FIG. 2. From a normal, 3-month-old, untreated virgin rat. Ducts, alveolar buds and early groupings of alveoli preparatory to lobule formation.

FIG. 3. From a 3-month-old, virgin rat, hypophysectomized, oöphorectomized and untreated for 10 days. Gland has regressed to a duct system.

FIG. 4. Same as in Figure 3, but treated with 6 mg. MH daily for 10 days. Maintenance of ducts and alveoli.

FIG. 5. Same as in Figure 3, but treated daily for 10 days with 1 μg. estrone, and three 12-day rat placentae. Fair L-A growth.

FIG. 6. Same, but treated daily for 7 days with 1 μg. estrone, 4 mg. progesterone, 2 mg. MH and 0.2 mg. STH. Then 1 mg. of hydrocortisone acetate (F) for 7 days. Gland regressed from L-A state.

FIG. 7. Same but treated daily for 6 days with 1 μg. estrone, 4 mg. progesterone and four 12-day rat placentae. Necropsy 1 day later. Fair L-A growth.

FIG. 8. Same as in Figure 7; but after the same treatment for 6 days, the rat was injected daily for 4 days with six 12-day rat placentae. Beginning lactation in a gland with fair L-A development.

FIG. 9. Same as in Figure 8 except that rat also received 1 mg. F during last 4 days. This is a 0.1 mm. thick section and shows fair lactation in a gland with good lobular structure.

FIG. 10. Same as in Figure 9 except that rat also received 0.5 mg. STH during last 4 days. This is also a 0.1 mm. section of a thick, fully developed gland engorged with milk.

TABLE 5. TESTS OF THE DIFFERENT PLACENTAL REGIONS FOR MAMMOTROPIC ACTIVITY

Series	No. of rats	L-A mammary growth	
		+†	−
Controls*	15	0	15
Whole Placenta	15	12	3
Decidua basalis	15	5	10
Träger plus Decidua capsularis	12	10	2
Träeger region	12	5	7
Decidua Capsularis region	15	10	5

* All rats were adult virgins, hypophysectomized and oöphorectomized. All received daily s.c. injections of 1 μg. estrone plus 4 mg. progesterone. The placental parts were 2% butanol suspensions of homogenates and all were given in doses of 3 placental equivalents.

† Most of the positive mammary reactions were in the 1+ range of lobular development.

the lower $\frac{1}{3}$ of the decidua capsularis including yolk sac and trophoblastic cells; and (2) the upper $\frac{2}{3}$ of the decidua capsularis, again with associated yolk sac endoderm and a scattering of giant trophoblastic cells. Homogenates were prepared from these separate parts; and injections were made into the same test animal as mentioned above in doses equivalent to 3 placentae.

Table 5 shows the results of these tests. The mammary glands of the rats receiving only estrone and progesterone showed regression. All of the groups injected with the various placental suspensions as well as estrone and progesterone showed some evidence of mammotropic activity. Probably the most significant figures were those showing that approximately the same response was obtained with tissue from the region listed in Table 5 as decidua capsularis as from that of the entire placenta. Such fragments weighed approximately 5 mg. or 10% of the entire placental weight.

DISCUSSION

Since it has been shown by Lyons (1937) that approximately 0.01 μg. of purified MH is capable of eliciting a detectable local growth response in the squab crop, it would appear that the 12-day rat placenta is not rich in this factor; or else substances capable of inhibiting the crop reaction were present in the crude extracts. From our earlier work (Lyons *et al.*, 1943b) (Averill *et al.*, 1950) it can be estimated that one 12-day placenta has the equivalent of 0.01 μg. of pituitary MH as determined by crop sac stimulating activity, but approximately 2 mg. of MH equivalent in terms of luteotropic activity. Since there has never been any chemical separation of crop-stimulating and luteotropic or mammotropic activities in the pituitary entity, it seems likely that the rat placental factor may be different in some respect from pituitary MH. Canivenc (1951) has also shown that the 12-day rat placenta implanted locally over the pigeon crop sac caused a proliferation of the epithelium qualitatively comparable to that induced by MH. His earlier work with Mayer (Mayer and Canivenc, 1950)

stressed some of the similarities between the placental substance and pituitary "prolactin." One must also be mindful of the experiments of Lahr *et al.* (1943) showing that in relatively high doses, various non-specific substances applied locally to the pigeon crop sac caused proliferation comparable to that which we are reporting for rat placenta. We have confirmed the results of Dresel (1935) with the finding (unpublished) that as low a daily dose as 0.1 mg. of MH in some mice and 0.25 mg. in some rats may prevent the appearance of vaginal cornification for 1 or 2 cycles. This test for MH is an easy one to perform; but a positive reaction in a normal animal is open to various interpretations (e.g., a substance such as testosterone may induce the pituitary to secrete MH and give the same end result). However, in the case of the rat placenta, its luteotropic activity was also determined in hypophysectomized animals.

In Experiment 2 an attempt was made to strike a correlation between the effect of the placenta on the estrous cycle and on the mammary gland. The cycle was inhibited presumably by over-secretion of progestin. Lobulo-alveolar mammary growth in such animals was thought to be due to an increase in progestin secretion, plus the action of direct mammary stimulating substances of the injected material and of the rat's pituitary. These results might be explained on the basis of a single substance similar to MH being formed in the placenta. This point was explored in further experimentation. It should be noted that the effects on the vagina and the mammary gland did not always coincide. Both of these organs are targets for estrin and progestin, but it should be recalled that the mammary gland requires other synergizing hormones, one source of which is the pituitary, another being the placenta.

In an earlier experiment (Lyons, 1950) it was noted that hypophysectomized-oöphorectomized pregnant rats maintained with estrone and progesterone showed good lobulo-alveolar mammary development at term. It seemed likely, then, that the placenta provided a mammotropic substance or substances that synergized with estrone and progesterone in much the same way as did MH and STH. This was proven in Experiment 3. Hormonal activity was demonstrable in the placentae of rats on their 8th day of gestation; or to use the more common dating method, 7 days after the detection of sperm. As explained above the term 8-day placenta refers to the time of gestation. If one were to restrict the term placenta to the fused maternal and fetal tissue, it would be necessary to credit the secretion of this mammotropic substance to a placenta barely formed. It seems likely that careful isolation, collection and testing of some of the earliest trophoblastic or yolk-sac structures of the pre-implantation stage will yield evidence of an early secretion of this hormone. The hypothesis of a luteotropic substance of blastocystic origin to account for a heightened activity of corpora lutea does not exclude the likelihood that pituitary MH is also important as a luteotropin at this stage. There are many ways of prolonging the luteal phase in the rat in the absence of the blastocyst,

but Experiment 3 showed that this early tissue may act hormonally, and not just as a physical agent that "stings" the endometrium, thereby setting up neural or indirect hormonal stimuli.

The 12-day placenta provided the best tissue for this investigation because of its high potency, and because it lends itself to easy dissection of its separate parts. The older placentae showed some activity, but on a weight basis were considerably less potent than the 12-day placentae. There was some indication that with increasing dosage of 12-day placentae a greater mammotropic effect was obtained. In some of the experiments we were dealing with variables such as the placentae used and the rats in which the mammary tests were read. Control of those would require a greater number of animals per level. Using purified pituitary MH with estrone and progesterone in the hypophysectomized-oöphorectomized rat it has been barely possible to attain a 1+L-A mammary development. Such animals customarily lost weight, and were in negative nitrogen balance. When STH, in a dose adequate to compensate for this deficiency was administered simultaneously, the mammary growth was equivalent to that of late pregnancy. It should be emphasized, therefore, that many of the rats injected with the higher doses of 12-day placenta and estrone and progesterone not only lost less weight but also showed better L-A growth than one sees with estrone, progesterone and pituitary MH. This suggests that the placenta secretes a different MH than the pituitary or that it possesses an anabolic hormone that imitates in some degree pituitary STH. Cole and Hart (1938) and Hart and Cole (1939) reported increased somatic growth rates in rats during pregnancy, pseudopregnancy and lactation; and noted a concurrent increase in appetite. As will be shown later the synergism of a substance like STH with MH is equally important in lactation.

One point was clarified in Experiment 4 namely, that in the rat with pituitary but not ovaries ablated, the placenta acts both as a direct and indirect mammary stimulator just as the pituitary MH does (Lyons *et al.*, 1943b). A new finding was that the placenta in a large enough dose could cause mammary growth in the hypophysectomized rat, without the help of added estrone or progesterone. Apparently one placenta is sufficient to stimulate the ovary to form approximately a 4 mg. equivalent of progesterone, but 5 are necessary to substitute for 1 μg. of estrone. The alternative explanation would be that the 5 placentae contained a gonadotropic substance comparable to the pituitary's FSH+ICSH combination found necessary in hypophysectomized rats to stimulate estrin secretion. Support for the idea that the 12-day rat placenta may contain small amounts of estrogenic and progestogenic substances as well as mammotropic activity was found in a short pilot experiment that has not as yet been extended. This showed that twelve 12-day placentae stimulated 1+L-A growth in 2 of 3 hypophysectomized-oöphorectomized rats.

It has been possible to demonstrate lactogenic activity in 12-day rat placentae by means of the guinea pig test. Due to recent clarification and substantiation in the rat of the Folley and Young[2] theory concerning the hormonal needs in lactation, it has also been possible to show placental lactogenic activity by employing a rat preparation currently in use in this laboratory. After inducing prolactational development of the hypophysectomized-oöphorectomized rat's mammary gland with estrone, progesterone plus MH+STH or rat placental extracts over a 6-day period it was possible to cause milk secretion with placental extracts, particularly when hydrocortisone and somatotropin were administered concurrently. The latter two hormones alone or combined do not initiate lactation. Further work is required to demonstrate what combination of substances in the placenta induces milk secretion without the aid of pituitary factors.

In judging relative potencies of the pituitary and rat placental mammotropic (luteotropic or lactogenic) hormones, difficulty is encountered because one has been isolated as a pure protein, and the other has not been characterized in any way except by the assumption that it is water soluble. The active substance must be highly concentrated in the "fetal" placenta since it has been shown the unextracted tissue is gravimetrically almost as potent as the pure pituitary MH. Further comparisons will have to await the outcome of attempts to study the placental hormone chemically.

Citations that go back to Hippocrates have stressed the lactation-inhibiting role of the placenta; and these usually pertain to term placentae of man and domestic animals. In all likelihood the content of estrin and progestin determine the lactation-inhibiting action of such placentae or placental remnants; and over and against these substances one must place the lactation-inducing hormones MH, STH, and ACTH of pituitary, and conceivably of placental origin. The titers of the activators versus the antagonists then become the deciding factors.

In Experiment 6 in which estrone, progesterone and placentae were used for 6 days to induce lobular development in the hypophysectomized-oöphorectomized rats, there was always a residue of the oil in which the steroids were dissolved at the injection sites during the 4 days of treatment with placentae alone, or in combination with hydrocortisone and/or STH. Rapid formation of milk in all of the rats so treated would indicate that the inhibitors may be "overridden"; as indeed they are in normal lactation in rats. Apparently the 12-day placenta was sufficiently potent in lactogenic substance(s) to override any inhibitor hormones as shown in rat 81, Table 5 (Fig. 8). It was found that the placental extracts were greatly

[2] That hormones other than the "classical" Stricker and Grueter (1928) mammary-stimulating substance (MH) deserve the term lactogenic or galactopoietic has been contended by Folley and co-workers for some time (Folley and Young, 1941). They believed that ACTH and STH were also important in lactation; and their contentions have been sustained.

helped by a small dose of adrenal steroid; and in this regard it should be stated that no evidence for an ACTH-like hormone in rat placenta as judged by adrenal weight change was found in our experiments. However, this is not sufficient evidence to rule out a placental ACTH. The possibility also remains that such placentae may possess adrenocorticoid activities similar to those found in human and equine placentae by Pearlman and Cerceo (1952) and Johnson and Haines (1952). Possession by the rat placenta of a combination of substances similar in actions to pituitary MH and STH and either ACTH or an adrenocorticoid would seem to be the best explanation for this organ in itself being lactogenic. This would also be useful in explaining the observations of Selye *et al.* (1933) and Newton and Beck (1939) in mice and rats, which, after having been hypophysectomized at mid-pregnancy, carried their fetuses to term, delivered them and secreted milk.

The problem of determining the number of rat placental hormones that synergize in mammary stimulation remains a difficult one. The cell responsible for the secretion of the MH-like substance is not known; but the evidence is presumptively in favor of the giant trophoblast cells. Mayer and Canivenc (1950) showed that rat placental autografts with prominent large trophoblastic cells were responsible for luteotropic, mammotropic and lactogenic effects.

SUMMARY

The rat placenta, at an early stage of development contains a substance, (or substances) with luteotropic, mammotropic, lactogenic, and crop sac-stimulating activities. Compared to pituitary mammotropin (prolactin), it is potent in the first three of these activities and weak in the last.

Luteotropic activity was shown by inducing a prolonged luteal phase in the rat ovary and thus inhibiting the estrus cycle. It was also shown by injecting estrone plus placenta into hypophysectomized rats to demonstrate growth changes in the mammary gland that may only be induced otherwise by pituitary MH plus estrin and progestin. This was also evidence for mammotropic (direct mammary stimulating) activity; but the animal preparation used more extensively for this purpose was the hypophysectomized-oöphorectomized rat given estrone, progesterone and placenta.

Placental lactogenic activity was demonstrated in hypophysectomized-oöphorectomized rats injected with estrone, progesterone and placenta to induce mammary growth and then with placenta with or without hydrocortisone. A better lactogenic combination was placenta plus hydrocortisone and pituitary somatotropin.

By using the highly sensitive local test it was possible to demonstrate crop sac stimulating activity in the equivalent of a single 12-day rat placenta.

Three parts of the 12-day rat placenta were tested separately for

mammotropic activities. All were active; but fragments of the decidua capsularis were especially potent. Wet weight equivalents of this tissue approximating 5 mg. showed activity corresponding to that of about 2 mg. of the purified pituitary mammotropin.

Acknowledgments

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REFERENCES

ASTWOOD, E. B. AND R. O. GREEP: *Proc. Soc. Exper. Biol. & Med.* **38:** 713. 1938.
ASTWOOD, E. B. AND R. O. GREEP: *Science* **89:** 31. 1939.
ASTWOOD, E. B.: *Endocrinology* **28:** 309. 1941.
AVERILL, S. C., E. W. RAY AND W. R. LYONS: *Proc. Soc. Exper. Biol. & Med.* **75:** 3. 1950.
CANIVENC, R.: *Arch. d'Anat. d'Hist. et d' Embryol.* **34:** 105. 1951.
COLE, H. H. AND G. H. HART: *Amer. Jour. Physiol.* **123:** 589. 1938.
DRESEL, J.: *Science.* **82:** 173. 1935.
FOLLEY, S. J. AND F. C. YOUNG: *Lancet.* **240:** 380. 1941.
HART, G. H. AND H. H. COLE: *Proc. Soc. Exper. Biol. & Med.* **41:** 310. 1939.
JOHNSON, R. H. AND W. J. HAINES: *Science.* **116:** 456. 1952.
LAHR, E. L., R. W. BATES AND O. RIDDLE: *Endocrinology* **32:** 251. 1943.
LYONS, W. R. AND E. PAGE: *Proc. Soc. Exper. Biol. & Med.* **32:** 1049. 1935.
LYONS, W. R.: *Cold Spring Harbor Symposia on Quantitative Biology.* **5:** 191. 1937.
LYONS, W. R.: *Endocrinology* **28:** 161. 1941.
LYONS, W. R.: *Essays in Biology, University of California Press.* Berkeley, 1943a.
LYONS, W. R., M. E. SIMPSON AND H. M. EVANS: *Proc. Soc. Exper. Biol. & Med.* **52:** 134. 1943b.
LYONS, W. R.: *Colloque International du CNRS: Mécanisme Physiologique de la Sécrétion Lactée.* Strasbourg, 1950.
LYONS, W. R., C. H. LI AND R. E. JOHNSON: *Jour. Clin. Endocrinol. & Metab.* **12:** (p. 45 Abstracts). 1952.
LYONS, W. R. AND R. E. JOHNSON: *Stain Technol.* **28:** 201. 1953a.
LYONS, W. R., C. H. LI, R. E. JOHNSON AND R. D. COLE: *Jour. Clin. Endocrinol. & Metab.* **13:** 836. 1953b.
MAYER, G. AND R. CANIVENC: *C. R. Soc. Biol.* **144:** 410. 1950.
NELSON, W. O. AND R. GAUNT: *Proc. Soc. Exper. Biol. & Med.* **34:** 671. 1936.
NEWTON, W. H. AND N. BECK: *Jour. Endocrinol.* **1:** 65. 1939.
PEARLMAN, W. H. AND E. CERCEO: *Jour. Biol. Chem.* **194:** 807. 1952.
RIDDLE, O., R. W. BATES AND S. W. DYKSHORN: *Proc. Soc. Exper. Biol. & Med.,* **29:** 1211. 1932.
SELYE, H., J. B. COLLIP AND D. L. THOMSON. *Proc. Soc. Exper. Biol. & Med.* **30:** 589. 1933.
STRICKER, P. AND F. GRUETER: *C. R. Soc. Biol.* **99:** 1978. 1928.

CHANGES IN THE GONADOTROPHS OF THE RAT PITUITARY AFTER GONADECTOMY

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IN A preceding paper (Purves and Griesbach, 1954) evidence was presented for the presence in the anterior pituitary of the rat of two specific types of gonadotrophic basophils. Of these, the peripherally situated, coarsely granulated type appeared to be concerned with the production of FSH, while the central, finely granulated type was assumed to produce LH.

In this paper, observations on gonadectomised rats will be reported, in which the resulting cell changes in the anterior pituitary are correlated to changes in the gonadotrophic potency of tissue from the same gland. It will be shown that the changes which occur in the follicle stimulating and luteinising potencies of the glands within certain times after gonadectomy, are paralleled by characteristic changes in the two types of gonadotrophic basophils. The evidence obtained by these observations supports the results of the preceding paper.

MATERIALS AND METHODS

Young mature male and female rats of a Wistar strain were gonadectomised and kept for varying times. The rats were killed after 10 days, 15 days, 21 days and 8, 16, 24 and 48 weeks. In each case the completeness of gonadectomy was verified by direct inspection and the evidence of regression of the uterus or the male accessories.

After removal, the pituitaries were divided with a razor blade into two approximately equal pieces. One part of the pituitary was fixed in formal-sublimate (1:9) for histological examination. The other part was used for assay of the gonadotrophic potency. In most experiments, two half pituitaries were combined for injection into one test animal, but in the sixteen and forty-eight weeks groups, one half pituitary only was used for the assay. The pituitary tissue to be assayed was ground up in a small agate mortar and suspended in 1 ml. of sterile saline. This amount was injected into littermate twenty-one day old female rats, in eight portions over four days. On the fifth day, the animals were killed and the gonads examined. Littermates were injected with suspensions of normal male rat pituitaries and others were retained as uninjected controls.

Histology

The pituitaries were cut at 2.5 μ and 5.0 μ. The sections were stained with Crossmon's modification of Mallory's triple stain (Crossmon, 1937), for general cytological information. The glycoprotein content of the pituitary cells was demonstrated by the periodic acid-Schiff process (PAS) following the description given in an earlier publication (Purves and Griesbach, 1951). The ovaries of the immature female rats were examined microscopically. One ovary of each animal was sectioned after paraffin embedding,

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and stained with haematoxylin-eosin, while frozen sections of the other ovary were stained for lipoids with oil red 0. (The solution used contained 20 mg. oil red 0 in 100 ml. of 60% isopropyl alcohol. This solution did not precipitate when kept in the incubator at 37° C.).

RESULTS

In Table 1, are summarised the results of experiments in which the gonadotrophic potencies were assayed and the cytology examined in separate halves of the same pituitary glands. These results have been supplemented by a large number of experiments in which gonadotrophic hormone assays and parallel cytological studies have been made on different pituitaries from groups of animals gonadectomised at the same time. The results of both types of experiment were similar, and are combined in the following statements:

Gonadotrophic potencies

The normal male pituitary has mainly a follicle stimulating effect. Only occasionally has some luteinising potency been observed. It will be seen that, 10 days after gonadectomy, the pituitaries of both sexes had a lower potency than the normal male's pituitary. At 15 days, the male castrate's pituitary was more potent than that of the normal male, but the potency of the female castrate was still below that of the normal male. At 10 days as well as at 15 days after gonadectomy, the pituitary extracts had a follicle stimulating quality only. At 21 days the gonadotrophic effects of the pituitaries of either sex were equal. The ovaries of the rats injected with extracts from 21 day castrate's pituitaries contained some corpora lutea, the main effect at this stage still being follicle stimulating. The pituitaries from rats of both sexes, gonadectomised for periods of 8 and 16 weeks, showed the greatest effect on the ovarian weights observed in this series. The ovaries consisted mainly of corpora lutea. At 24 weeks, the test ovaries were not as large as those at 8 and 16 weeks and the number of corpora lutea was reduced. At 48 weeks, the ovaries contained no corpora lutea but showed an increased number of enlarged follicles.

Pituitary cytology

The cell changes in the rat pituitary after gonadectomy which have been known for 50 years will be described here under the assumption that, after gonadectomy, two independent types of "castration cells" come into existence which in our opinion are derived from the two types of gonadotrophs described by us in normal animals (Purves and Griesbach, 1952, 1954). According to their regional distribution within the anterior lobe, they are referred to in this description, and also in Figures 1–21, as "peripheral" and "central" cells.

At 10 days after gonadectomy, the gonadotrophs of both types were slightly increased in numbers and size. The peripheral cells contained some

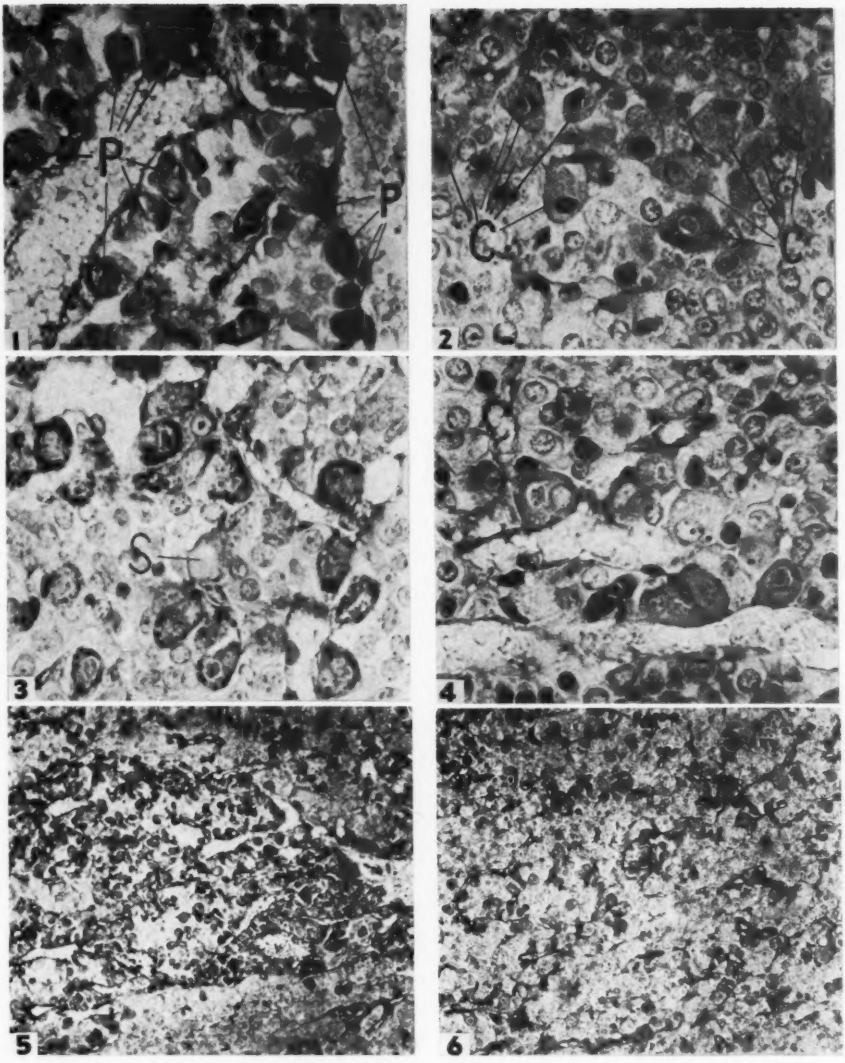
TABLE 1

Donor rats	Time after gonadectomy	Assay of rat pituitaries for gonadotrophic hormones. Response in assay animals			Cytology of castrate rat pituitaries	
		Ovaries		Uterus	FSH cells	LH cells
		Weight, mg.	Histology	Weight, mg.		
A Castrate Males	10 days	31.5; 36.6	Follicles only	71.0; 64.0	Increase in size. Loss of granules	Increase in size. Cells are quite pale
	15 days	43.6; 40.6; 39.6	Follicles only	97.9; 77.9; 69—9	Increase in numbers and size. Stronger granulation	Increase in numbers. Cells still quite pale
	21 days	32.4; 56.3	Follicles. Some corpora lutea	98.4; 99.9	No further increase in numbers	Large increase in numbers, size and glycoprotein content
	8 weeks	59.4	Follicles. Many corpora lutea	76.8	Onset of vesiculation. Some typical signet ring cells	Glycoprotein storage round golgi body
	*16 weeks	50.5; 42.5	Follicles. Many corpora lutea	72.0; 88.3	Vesiculation prevalent. Dark, coarse granulation	Many vesiculated cells. Some cells densely granulated, others only lightly granulated
	24 weeks	40.5	Follicles. Some corpora lutea	112.9	Some regressive changes especially in signet ring cells	Advanced regression. Loss of glycoprotein content. Cytoplasmic vacuolation
	*48 weeks	44.2; 62.5	Follicles only	99.6; 97.5	Recovery. Numerous densely granulated small cells in periphery. Few signet rings	Only remnants of cells and vesicles visible
B Spayed Females	10 days	24.4; 28.8	Follicles only	37.8; 29.5	Similar appearance to male at 10 days, but smaller number of cells	Similar appearance to male at 10 days, but smaller number of cells
	15 days	22.0; 29.3; 31.8	Follicles only	48.5; 54.2; 59.5	Similar appearance to male at 15 days, but smaller number of cells	Similar appearance to male at 15 days, but smaller number of cells
	21 days	36.4; 36.7	Follicles. Some corpora lutea	64.4; 83.1	Similar appearance and number to male at 21 days	Similar appearance and number to male at 21 days
	8 weeks	66.6	Follicles. Many corpora lutea	75.0	Similar appearance and number to male at 8 weeks	Similar appearance and number to male at 8 weeks
	*16 weeks	57.8; 58.0	Follicles. Many corpora lutea	100.9; 84.7	Similar appearance and number to male at 16 weeks	Similar appearance and number to male at 16 weeks
	24 weeks	39.6	Follicles. Some corpora lutea	85.8	Similar appearance and number to male at 24 weeks	Similar appearance and number to male at 24 weeks
	*48 weeks	29.3; 45.0	Follicles only	44.2; 99.6	Similar appearance and number to male at 48 weeks	Similar appearance and number to male at 48 weeks
C Normal Males 17 rats 3–6 months old		†36.49 ± 1.76	Follicles only	†82.06 ± 8.35		
D Uninjected Assay controls 13 rats 25 days old		†16.71 ± 0.46	Immature	†25.53 ± 1.16		

* One half pituitary injected into assay rats.
† Mean and S.E.

of their characteristic coarse, dark granules, but apparently not as many as under "normal" conditions (Fig. 1). The central cells were so poor in glycoprotein content that photography met with difficulties. Obviously both types of cells were losing hormones at this stage (Figs. 3 and 4).

At 15 days, the pituitaries of the males showed a large increase in strongly granulated peripheral gonadotrophs which spread along the portal vessels in great numbers (Figs. 5, 7, and 21b). The central cells, though also increased in numbers, were restricted to small patches in the centre (Figs.



DESCRIPTION OF PLATES

All plates are photomicrographs of sections of rat pituitaries stained by PAS and haematoxylin.

FIG. 1. Peripheral gonadotrophs of normal adult male rat showing dense cytoplasmic glycoprotein granules (P). ×480.

FIG. 2. Central gonadotrophs of normal adult male rat showing a pale cytoplasmic glycoprotein reaction due to a fine, scanty granulation (C). ×480.

FIG. 3. Peripheral gonadotrophs of male rat 10 days after castration, showing various degrees of degranulation. The remaining coarse granules tend to be concentrated at the periphery of the cell. A "signet-ring" cell (S), which must have been present before castration, is seen in the centre of the field. ×480.

FIG. 4. Central gonadotrophs of male rat 10 days after castration. The cells are enlarged, and the negative image of the golgi body is visible. The glycoprotein reaction is still weak. ×480.

6 and 8). The gonadotrophs in the female did not show the same increase in numbers.

At 21 days, the peripheral cells remained as at 15 days, but the central cells showed a rise in numbers and size (Figs. 9, 10, and 21c). Some of these cells were quite large and contained two nuclei and greatly enlarged golgi bodies.

At 8 weeks, a few peripheral cells showed vesiculation and formation of "signet rings," with storage of glycoprotein in the periphery. The central cells, some of which also showed vesicles at this stage, had dark glycoprotein granules concentrated around the golgi body making the negative image of this structure especially conspicuous at this stage (Figs. 11, 12, and 21d).

At 16 weeks, the "castration" changes had reached a maximum in cell numbers, vesiculation of both cell types, and hormone storage as indicated by the strength of the glycoprotein reaction (Figs. 13 and 14). After this period a cell regression was observed which was most obvious in the central cells but affected the peripheral cells also.

At 24 weeks many of the peripheral cells showed indistinct or irregular features with loosening up of the cytoplasmic structure, widening of the intergranular spaces and pyknosis of the nucleus. In addition to these, some smaller healthy looking cells of the peripheral type could also be found in such sections. In the central area, the numbers of typical central gonado-

FIG. 5. Peripheral gonadotrophs of male rat 15 days after castration, showing large numbers of cells present, especially along the portal vessels at the anterior border of the gland. ×107.

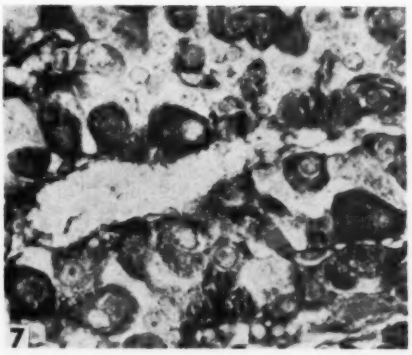
FIG. 6. Central gonadotrophs of male rat 15 days after castration showing a moderate increase in the numbers of these cells, which become much more numerous at a later stage. ×107.

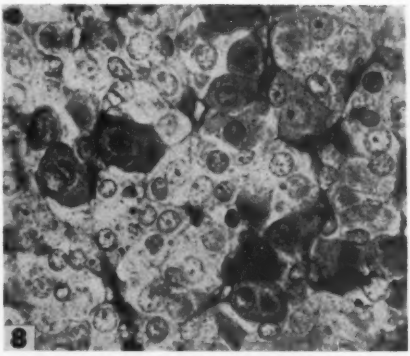
FIG. 7. Same section as Figures 5 and 6. Peripheral gonadotrophs showing varying degrees of degranulation. ×480.

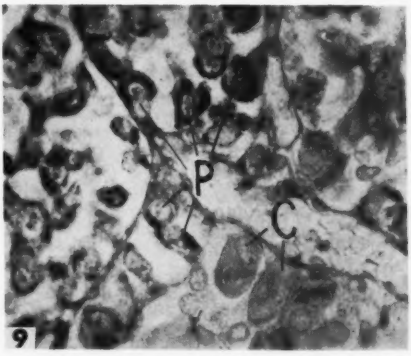
FIG. 8. Same section as Figures 5 and 6, showing central gonadotrophs. The cytoplasmic glycoprotein reaction is still relatively weak. ×480.

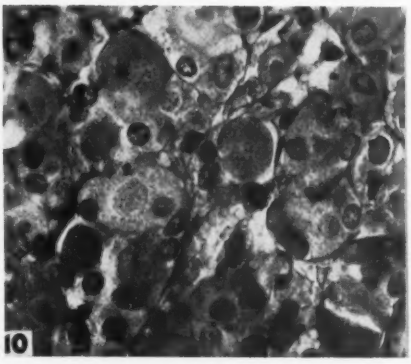
FIG. 9. Field showing both peripheral and central type of gonadotroph cells. Pituitary of male rat 21 days after castration. The appearance of the peripheral type (P) is similar to that at 15 days with varying degrees of degranulation. The granules are coarse and darkly staining. Two cells of the central gonadotrophic type (C) are adjacent to the wall of the blood vessel. Their granulation is finer and palely stained and is superimposed on the diffuse pink cytoplasmic coloration. ×480.

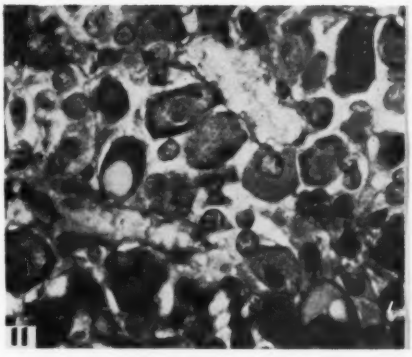
FIG. 10. Central gonadotrophs of male rat 21 days after castration. The numbers of these cells has increased markedly as compared with the 15 day pituitary. The glycoprotein reaction has also increased in intensity. Some binucleate cells are present. ×480.











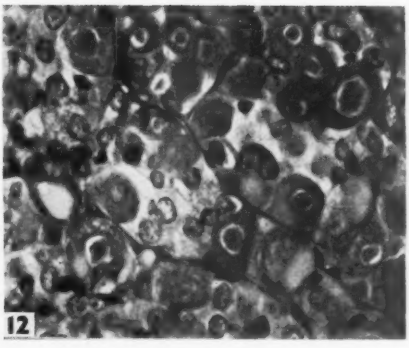
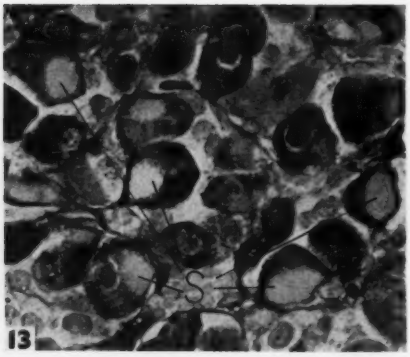


FIG. 11. Peripheral gonadotrophs of male rat 8 weeks after castration. Vesiculation has occurred in some of the cells producing "signet-ring" cells. ×480.

FIG. 12. Central gonadotrophs of male rat 8 weeks after castration. In addition to the diffuse, fine, pink granulation there is an accumulation of coarser glycoprotein granules both within and around the golgi body rendering the negative image of the golgi more conspicuous. ×480.



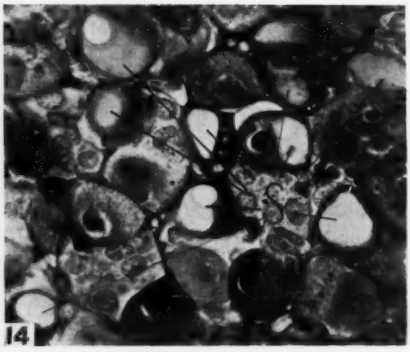


FIG. 13. Peripheral gonadotrophs of male rat 16 weeks after castration. A high proportion of "signet-ring" cells is present. The vesiculation has enlarged the cells but has not yet compressed the peripheral cytoplasm, so that at this stage the "signet-ring" cells are seen in their most typical form (S). The cells are uniformly granulated with coarse dark granules. ×480.

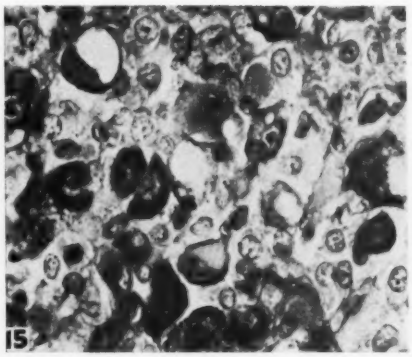
FIG. 14. Central gonadotrophs of male rat 16 weeks after castration. Vesiculation leading to the formation of "signet-ring" cells has occurred in a proportion of the central gonadotrophs (S). Both the vesiculated and non-vesiculated cells show a varying intensity of glycoprotein reaction from quite pale up to very dense reactions. There is a tendency for the glycoprotein reaction to be strongest in and around the golgi body and for the peripheral cytoplasm to be relatively pale. ×480.

trophs were much reduced and in their place areas of large degenerating pale cells could be seen, with ill defined nuclei and vacuolated cytoplasm (Figs. 15 and 16).

At 48 weeks the peripheral cells were present in a variety of size and activity, and an impression was gained that a regeneration of this cell type had taken place. In contrast, the centre contained some small pink cells and hyaline masses representing remnants of former signet ring cells (Figs. 17, 18, and 21e). Eighteen months after gonadectomy we found similar changes which had advanced even further towards annihilation of the central type (Figs. 19 and 20).

Correlation between gonadotrophic potencies and cytological changes

It was noticed that, after gonadectomy, changes in the anterior pituitary of the rat take place which are progressive and reach a static stage after approximately one year. These changes concern the quantity and quality of the gonadotrophic potency as well as those of the gonadotrophic basophil cells. We have concluded that a correlation exists between the prevailing gonadotrophic quality at a certain time after gonadectomy, and the basophil cell type which is dominant at the same time. For the first 2 weeks after gonadectomy the pituitaries contain mainly FSH. At this time most



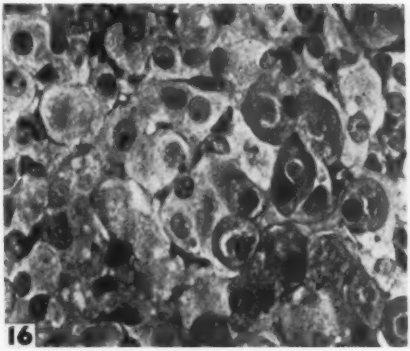
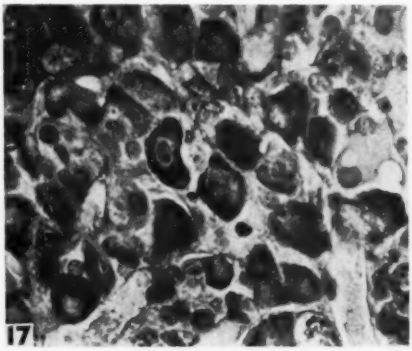


FIG. 15. Peripheral gonadotrophs of male rat 24 weeks after castration. There is a marked regression in both numbers and size compared with the picture at 16 weeks. The "signet-ring" cells show a thinning of the cytoplasmic wall of the vesicle. Some cells seem to be losing their granulation. ×480.

FIG. 16. Central gonadotrophs of female rat 24 weeks after castration. There has been a reduction in both size and glycoprotein content of these cells since the 16 week period. Some cells can be seen which have lost almost all their glycoprotein, others still retain glycoprotein but are reduced in size. ×480.

of the increased basophils are of the peripheral type. From 3 to 24 weeks, the pituitaries have strongly luteinising properties which show a maximum between 8 and 16 weeks and decline slowly later on. During this period the



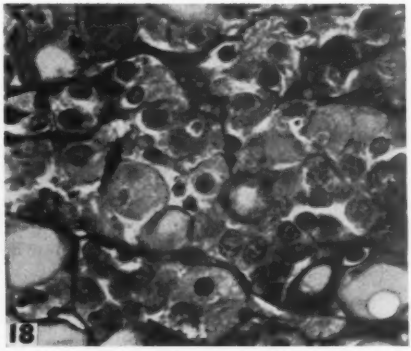
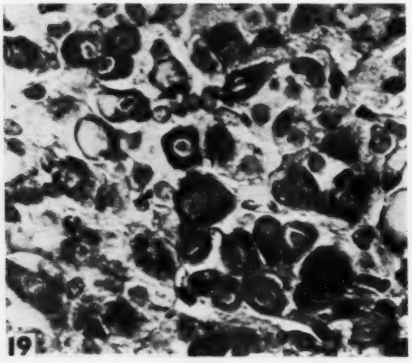


FIG. 17. Pituitary of male rat 48 weeks after castration. The peripheral gonadotrophs are small and inactive-looking, but are present in large numbers and have a strong glycoprotein granulation. Most of these cells are not vesiculated, a few remnants of vesiculated cells which have lost almost all their cytoplasm being present. ×480.

FIG. 18. Central gonadotrophs of male rat 48 weeks after castration. There has been an almost complete regression of this type of cell. Such as are still visible show only a faint pink glycoprotein reaction. Remnants of former vesiculated cells persist, with only scanty cytoplasm and weak glycoprotein reaction. ×480.



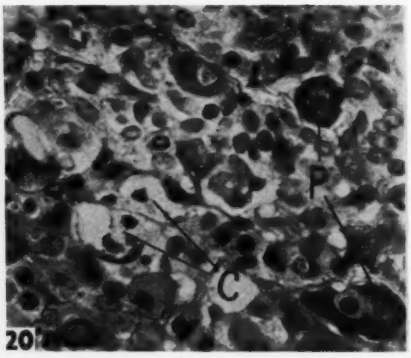


FIG. 19. Peripheral gonadotrophs of male rat 18 months after castration. Small inactive-looking gonadotrophs with strong glycoprotein granulation are present in considerable numbers, with a few "signet-ring" cell remnants with scanty cytoplasm persisting. ×480.

FIG. 20. Central gonadotrophs of male rat 18 months after castration. The degeneration of this type of cell is at this time complete. The only signs of their former presence is given by some large vesicles considered to be the remnants of former "signet-ring" cells and some small pale cells with pyknotic nuclei (C). The darkly granulated cells visible in this field are peripheral type gonadotrophs (P), the field having been chosen from the region in which the distributions of the two types overlap. ×480.

overwhelming majority of basophils are of the central type, which at this time have their maximum size and glycoprotein content. At a later stage, about 12 to 18 months after gonadectomy, when the pituitary gonadotrophic potency is follicle stimulating, the basophil cells are overwhelmingly of the peripheral type. The central cells have degenerated and only remnants of these cells can be found.

We conclude that the presence of the central cell type is linked with the presence of luteinising hormone, while the peripheral cells are responsible for the follicle stimulating effect.

DISCUSSION

Large cells of abnormal appearance in the pituitary of the castrate rat were apparently first observed by Zacherl (1913) who interpreted these cells as being transformed acidophils. Schleidt (1914) coined the term "signet ring cell" to describe the vacuolated castration cell in the rat pituitary. Addison (1917) found that the castration cells in the rat were enlarged basophils and the basophil nature of castration cells has been recognised by most subsequent investigators. The presence of glycoprotein in castration cells in the rat was first demonstrated by Catchpole (1949).

There is evidence that earlier observers have observed the characteristic difference in granulation in the two specific types of castration cell. Addison (1917) observed in rats, seven months after castration, among non-

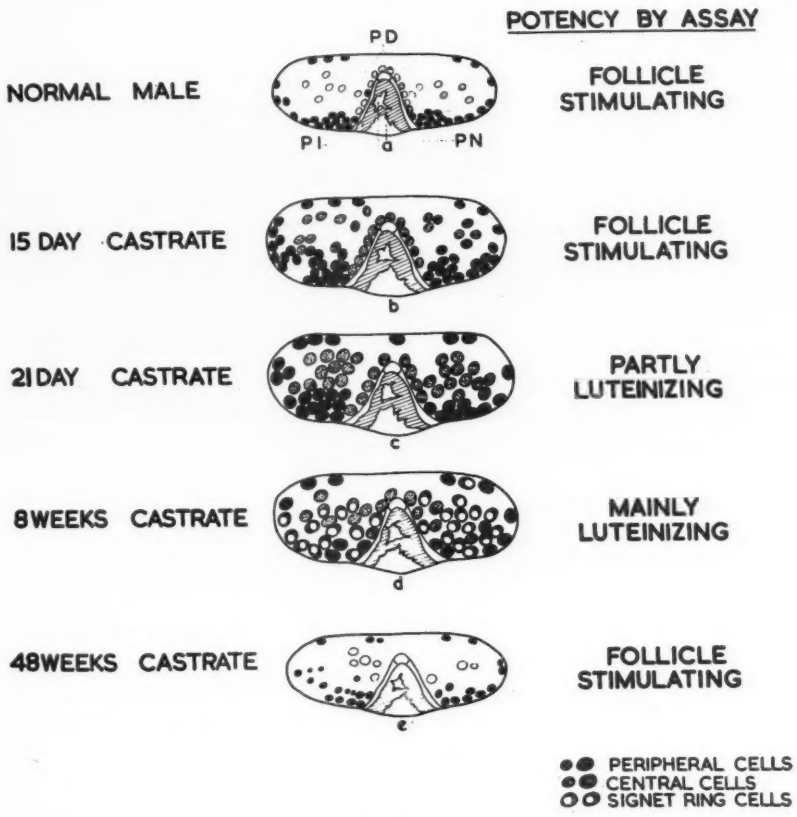


Fig. 21 a-e

FIG. 21 a–e. Diagram showing the relative numbers and the distribution of the two types of gonadotrophs at varying times after castration. The gonadotrophic qualities preponderant at the same time intervals have been indicated.

vacuolated basophils two varieties, some coarsely granulated, others finely granulated. The same two varieties were observed by him in the normal male rat. Severinghaus (1933) also recognised a variation of granulation in the basophils of the rat pituitary, some being finely granulated, some coarsely granulated and some floccularly granulated. The fact that basophil cells with different types of granulation are differently distributed within the anterior lobe does not seem to have been recognised by these workers.

Recently Farquhar and Rinehart (1954), by means of electron microscope studies, have revealed many details of the structure of the basophil cells of the rat pituitary which cannot be visualised by ordinary microscopic techniques. They find that the castration cells of the rat pituitary

may be divided into two distinct types, and consider that these probably represent the two types of gonadotrophic basophil which we have described.

The acidophil cells appear to undergo a decrease in size and numbers after castration in the rat (Severinghaus, 1933). There is, therefore, no correlation between the acidophil cells and the increased potency of the pituitary in gonadotrophic hormones.

The hyaline material which appears in the vesicles of a proportion of the castration basophils and transforms them into "signet rings," is of unknown significance. Signet ring forms appear only 8 weeks after castration, by which time the increased gonadotrophic potency and the strongly luteinising quality is already marked. The further accumulation of a substance within signet ring cells which continues for another 4 months, is not paralleled by any increase in either follicle stimulating or luteinising potency. The hyaline substance does not, therefore, appear to be gonadotrophin. This is in accordance with the absence of a glycoprotein reaction, as described by Pearse (1953). The changes in gonadotrophic potency occurring in the rat pituitary after castration are, therefore, to be correlated with the intensity of the cytoplasmic glycoprotein reaction in specific gonadotrophin producing cells.

Hellbaum and Greep (1940) have extensively investigated the changes in gonadotrophic potency in the male rat pituitary after castration. Their results with the assay of the pituitaries of male rats castrated as adults are closely paralleled by our own results. The initial depression of gonadotrophic potency at ten days is evident in their results. In their experiments, luteinisation of test ovaries was produced in a proportion of the responses after 15 days castration, whereas in our animals this response was not observed until 21 days post-castration. After 20 days and up to 9 months following castration the pituitaries invariably induce the formation of corpora lutea in hypophysectomised recipients in Hellbaum and Greep's experiments; in normal recipients this luteinising ability appeared to persist for some longer post-castration interval. The luteinising ability then disappeared, and only a follicle stimulating effect was obtained. Our own results are similar to these. As Hellbaum and Greep have shown, the rate of progression of the changes in the pituitary gonadotrophic complex through a strongly luteinising phase to a purely follicle stimulating content depends upon the age at which the castration is performed.

Although all our assays have been performed on non-hypophysectomised immature female rats, the results of Hellbaum and Greep with parallel assays on hypophysectomised and non-hypophysectomised recipients appear to establish the validity of the use of non-hypophysectomised animals for the demonstration of changes in the gonadotrophic complex after castration.

The lower gonadotrophic potency of the mature female rat pituitary has

long been known. In our assays of the ovariectomised mature female rat pituitary, the lower gonadotrophic potency of the female as compared with the male appeared to persist for about 15 days. Thereafter there did not seem to be any quantitative or qualitative differences in the gonadotrophic effects produced by pituitaries of ovariectomised females compared with castrate males.

The correlation of the changes in gonadotrophic potency with variations in the glycoprotein content of the two gonadotrophic cell types is in accordance with the view previously expressed that the peripheral gonadotrophic cell type is responsible for the secretion of follicle stimulating hormone only, while the central gonadotrophic type is responsible for the luteinising hormone. The normal adult male pituitary is predominantly follicle stimulating, although it occasionally produced luteinisation of test animals' ovaries. The deep staining of the granules of the peripheral gonadotrophs by the PAS method for glycoprotein is in accordance with this predominantly follicle stimulating action. On the other hand, the central gonadotrophs give only a pale pink coloration with the PAS staining. This coloration is easily masked by counterstaining if too strong a nuclear stain is used, and this accounts for the presence of this type in normal pituitaries being previously overlooked.

The immediate fall in gonadotrophic potency after castration is revealed histochemically as a weak granulation of the peripheral gonadotrophs, presumably the result of rapid discharge of follicle stimulating hormone.

The great increase in potency which occurs from about 21 days up to 4 months after castration is seen to be due to the formation and activation of large numbers of gonadotrophs of both types and to an increasing accumulation of glycoprotein within their cytoplasm. The glycoprotein of the central gonadotrophic type is increased much more, relative to the normal state, than that of the peripheral type since there is not only a vast increase in the numbers of this type of cell containing glycoprotein, but also a strikingly increased depth of glycoprotein reaction in these cells which in the normal state give only a weak reaction. In this way, the predominantly luteinising action of the fully developed castration pituitary is explainable.

SUMMARY

Pituitaries of rats, gonadectomised for varying lengths of time, have been examined for their gonadotrophic potencies and for their histological changes.

As described by earlier workers, a progressive change in the quality of the gonadotrophic complex takes place, which during the first 15 days is predominantly follicle stimulating, then passes through a luteinising phase for approximately 9 months and is once more follicle stimulating from then on.

The basophils, appearing in large numbers in the anterior pituitary after

gonadectomy, may, on the basis of their glycoprotein content and their localisation within the pituitary, be divided into two independent groups which correspond to the peripheral and central types described in the normal rat.

There is a progressive change in the preponderance of either of these two cell types after gonadectomy, which closely parallels the qualitative change in the gonadotrophic potency. The largest number of central cells is present during the luteinising phase, while the peripheral cells are representative of the follicle stimulating phase. It is suggested that the peripheral cells produce the follicle stimulating hormone, while the central cells are responsible for the luteinising hormone.

REFERENCES

ADDISON, W. H. F.: *J. Comp. Neurol.* **28**: 441. 1917.
CATCHPOLE, H. R.: *J. Endocrinol.* **6**: 218. 1949.
CROSSMON, G.: *Anat. Rec.* **69**: 33. 1937.
FARQUHAR, M. G. AND J. F. RINEHART: *Endocrinology* **54**: 516. 1954.
HELLBAUM, A. A. AND R. O. GREEP: *Am. J. Anat.* **67**: 287. 1940.
PEARSE, A. G. E.: *J. Path. & Bact.* **65**: 355. 1953.
PURVES, H. D. AND W. E. GRIESBACH: *Endocrinology* **49**: 264. 1951.
PURVES, H. D. AND W. E. GRIESBACH: *Proc. Univ. Otago Med. School* **30**: 27. 1952.
PURVES, H. D. AND W. E. GRIESBACH: *Endocrinology* **55**: 785. 1954.
SCHLEIDT, J.: *Zentralbl. Physiol.* **27**: 1170. 1914.
SEVERINGHAUS, A. E.: *Anat. Rec.* **57**: 149. 1933.
ZACHERL, H.: Quoted from A. Biedl: Innere Sekretion, Vol. 2., p. 107, 1913. Urban & Schwarzenberg, Vienna.

ROLE OF IODINE DEFICIENCY IN THE PRODUCTION OF GOITER BY THE REMINGTON DIET

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OVER a hundred years ago Prevost (1830) suggested that a deficiency of iodine was responsible for goiter. This hypothesis has since been supported by the following pieces of evidence: 1) in many areas where the incidence of goiter is high, the iodine content of the soil, food and drinking water is low[3]; 2) the daily output of iodine in the urine of individuals residing in such areas is also low and, since these people may be considered to be in balance with their environment as regards iodine, it follows that their iodine intake also is low (von Fellenberg, 1923–1930; Lunde, 1928; Stanbury *et al.*, 1952); 3) the thyroid glands of individuals in goiter districts usually have a low iodine concentration[4] and an increased avidity for iodine (Stanbury *et al.*, 1952); 4) the administration of iodine in small amounts reduces the incidence of endemic goiter in men and animals.[5]

Experimental evidence in support of the iodine deficiency hypothesis of goiter was obtained by Remington and his collaborators (1936, 1937b). They found that goiter could be readily produced by feeding rats on a low iodine diet. The size of the thyroid was inversely related to the amount of iodine present in the diet.

In spite of this impressive array of evidence in favor of the iodine deficiency hypothesis, serious objections have been raised. Greenwald (1946), perhaps the most enthusiastic opponent of this theory, denied that iodine deficiency was the cause of either endemic goiter or experimentally produced goiter. He pointed out that: 1) certain low iodine diets do not pro-

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[3] Boussingault, 1831, 33; Chatin, 1850; von Fellenberg, 1923 et seq.; McClendon, 1923, 1927, 1933; McClendon and Williams, 1923 a, b; McClendon and Hathaway, 1924; Hercus, Benson and Carter, 1925; Hercus and Roberts, 1927; Mazocco, 1929; Bodnar and Straub, 1930; Adolph and Chen, 1930; Adolph and Whang, 1932.

[4] Oswald, 1897; von Rositzky, 1897; Morgenstern, 1912, Mueller, 1923 (quoted by Greenwald, 1946).

[5] Marine and Lenhart, 1910, 1911; Marine and Kimball, 1917, 1920, 1921; Kimball, 1928 a, b; Kimball and Marine, 1918; Kimball, Rogoff, and Marine, 1919; Marine, 1935; Klinger, 1921; Silberschmidt, 1927; Nicolaysen, 1927; von Jauregg, 1927; Muggia, 1927; Jones, 1928; Abbott, 1932.

duce goiter; 2) those that do produce goiter may be effective for reasons other than their low iodine content.

The first objection will be discussed later. The second objection derived some support from the discovery of a number of goitrogenic substances, the "anti-thyroid drugs," and particularly from the finding in natural foods of such a substance, 1-5-vinyl-2-thiooxazolidone (Astwood *et al.*, 1949), that inhibited thyroid function in man or rats fed diets that were not low in iodine. The concentration of this compound in rape seed was considered adequate to account for all the goitrogenic activity of this food (Astwood, 1949). Similar goitrogenic compounds might be responsible for the production of goiters previously attributed to the lack of iodine. In fact, Greenwald (1946) suspected that Remington's Diet might contain such a "positive goitrogenic factor".

The present experiments were devised to determine whether a positive goitrogenic factor was present in the Remington Diet No. 342 (Remington, 1937b). This diet lent itself well to this type of work, because of its relatively simple composition and low iodine content. Furthermore, it has been used routinely in a slightly modified form in this laboratory for the induction of goiter in rats and mice.

The method used to search for a positive goitrogenic factor in anyone of the foodstuffs composing the Remington diet was to investigate whether or not thyroid enlargement could still be obtained when the foodstuff was omitted from the diet.

Of all the components of this diet only sodium chloride was found to have an important specific effect on the thyroid gland. A quantitative experiment was then carried out in order to determine how the level of iodine affected the structure and function of the thyroid gland, that is, the production of goiter, in mice receiving graded amounts of sodium chloride. The relation between the effects of iodine and sodium chloride was thus clarified.

MATERIALS AND METHODS

Mice were selected as test animals for all the experiments because of the responsiveness of their thyroids to the Remington Diet, and the ease with which large numbers of these animals could be handled. An inbred strain was used to insure uniformity of genetic background.

Immature C3H (Heston) male mice, weighing 14 to 18 gm., were distributed at random in groups of between 10 and 18 animals, 4 to 7 to a cage. The basal diet used as control in most of the experiments was a modification of the Remington Diet, the composition of which is shown in Tables 1 to 5 opposite Group A in each case. The foodstuffs were obtained from the following sources: corn meal—Cotton Bob-o-link Brand Maple Leaf Milling Co., Montreal; liver—Extracted Liver Residue, Wilson Laboratories, Chicago; yeast—Dried Brewer's Yeast, Dow Kingsbeer Breweries, Montreal; wheat gluten—Industrial Grain Products Ltd., Starch and Chemical Division, Ogilvie Flour Mills Co. Ltd., Montreal (Wheat Gluten was obtained as granules and, therefore had to be ground in a ball mill before being used; for some experiments the gluten was washed to

remove the abundant sodium chloride present, dried and reground); glucose, calcium carbonate, and sodium chloride were reagent grade chemicals.

The foodstuffs were blended in powder form in a Hobart mixer. When a component such as corn meal was omitted from the experimental diet (Table 1), appropriate substitutes were included, e.g. glucose to supply the necessary carbohydrate, and liver to supply protein. Each new batch of foodstuff or chemically pure salt used in the diet was analysed for iodine content in sample lots of 250 mg. by a modified Chaney procedure (Gross, 1949). The large variations in iodine values (Tables 1 to 6) were due in part to the fact that several batches of food had to be used in some experiments and in part to the poor reliability of the iodine method for small samples having very low iodine levels. In the last experiments, the situation was improved by carrying out iodine determinations on larger quantities of food. This required predigestion of the food samples to reduce the amount of organic material by calcination in the presence of an alkali, followed by the modified Chaney procedure (Isler, unpublished). The values for the iodine content of the whole diets in Table 8 were thus obtained and are more reliable than those in the earlier tables.

The sodium content of foodstuffs was determined by flame photometry after digestion in concentrated nitric acid. The procedure of Wilson and Ball (1928) was used for the chloride determinations.

The diets were administered in open glass cold-cream jars fitted with wire mesh discs which rested on top of the food—an arrangement reducing wastage. Distilled water was given *ad libitum*. The cages were kept in a room where the temperature was maintained between 75 and 85° F. and artificial light was automatically turned on from 8:00 A.M. to 8:00 P.M.

After the diets had been fed for 50 to 70 days, each animal was weighed and given 2 μc. of radio-iodine in distilled water subcutaneously. Exactly $2\frac{1}{2}$ hours later, during which period all food was withdrawn but water was allowed, the animal was sacrificed with chloroform. The left thyroid lobe was weighed and dissolved in 2N NaOH preparatory to counting under a Geiger-Mueller tube; and the right thyroid lobe still attached to the trachea was placed in Bouin fluid, care being taken to avoid trauma to the gland during these steps. Sections were cut at 6 μ and stained with haematoxylin and eosin, Masson trichrome, or the PA-Schiff technique. In addition, stained and unstained radioautographs were prepared.

The Quantitative Experiment

109 C3H (Heston) male mice, aged 3 to 4 months and kept in a controlled temperature room (80° $\pm$5° F.), were fed a control low iodine diet consisting of glucose, washed wheat gluten, yeast and calcium carbonate which contained 4.1 μg. of iodine/100 gm. of diet (as estimated by the more accurate method). The mice were divided into four groups; the first was fed the control diet without added iodine; the three other groups received added amounts of potassium iodide, raising the iodine levels to 12.2, 32, and 100 μg./100 gm. of diet respectively. Each of these four groups was further divided into four subgroups receiving 0, 0.1, 1.0, and 3.0 gm. of added sodium chloride per 100 gm. of the respective diets. Sodium chloride replaced the same amount of glucose.

Iodide was added by spraying the required amount of potassium iodide solution onto the glucose. After being allowed to dry at room temperature, the sprayed glucose was mixed with the other constituents of the diet. Sodium chloride was mixed into the diet as a powder, except at the 0.1% level, in which case an aqueous solution of sodium chloride was sprayed onto the glucose in the same manner as for addition of iodide. Otherwise, the experimental conditions were the same as described previously.

The mice were sacrificed on the 68th and 69th days after being placed on the diets.

During the last 3 days of the experiment, food intakes were measured. The calculations of iodine and sodium chloride intake were based on the average food intake for these 3 days for all the animals in the experiment (3.16 gm./mouse/day).

Mode of presentation of results

The table summarizing each assay gives the composition of the diet used; the foodstuffs which have been changed are reported in italics. The results of the assay, that is, body weights, thyroid weights, radio-iodine uptakes and, in table 8, the iodine content of the diets are presented with their standard errors. The experimental values are underlined when they differ significantly from those of the control groups (which are referred to as Groups A or A').

To determine the thyroid weights and radio-iodine uptakes, only the left lobes were used. It was eventually realized that the enlargement of the left and right thyroid lobes in mice fed the low iodine diet was not identical. Thus in an experiment where both lobes were compared in 12 mice after 56 days on the basal modified Remington Diet, the average difference between the left and the right lobe weights was 1.13 mg., that is, the weight of the left lobe exceeded by 17.7% the mean lobe weight obtained by averaging right and left lobes (6.35 mg.). In 8 of the 12 thyroids examined, the left lobe was larger than the right, in 1 they were equal, and in 3 the right was larger than the left. (The greatest single difference was found in an animal in which the left lobe was greater than the right one by 51%). On the other hand, of 601 animals autopsied, 2 had no right thyroid lobe and 4 had no left lobe. These animals were not included in any of the calculations.

Examination of Tables 1, 2 and 8 shows several cases in which the I^{131} uptake in the left thyroid lobe is greater than 50%. Such an apparently illogical finding may be explained by the fact that at $2\frac{1}{2}$ hours after injection, the loss of radio-iodine in the urine is of the order of only 2 to 3%, whereas almost all the rest is fixed in the thyroid; therefore, the inequality in average lobe size accounts for the excessive per cent uptake in the left lobe.

RESULTS

Role played by the individual components of the Remington Diet in the production of goiter in mice

Corn Meal (Table 1). Control animals received the basic diet including corn meal (Group A). Either glucose with liver as a source of protein (Group B), or glucose alone (Group C) were used as substitutes for the corn meal.

TABLE 1. ROLE OF *corn meal* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: 59 Days)

Group	Per cent composition of diet							Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl	Glucose		Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	*72*	—	16	10	1	1	—	4.0 (2.3–6.4)	16	16	22.3	5.7±0.7	5.4±1.2	43.0±3.8
B	—	*10*	16	10	1	1	*62*	3.5 (2.3–5.2)	16	11	21.9	4.9±1.0	10.0±1.4	51.2±2.2
C	—	—	16	10	1	1	*72*	1.7 (0.8–2.6)	16	14	21.3	4.6±0.8	9.2±1.3	42.9±3.0

TABLE 2. ROLE OF *liver* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: Group A, B, 52 Days; Group A′, B′, 97 Days)

Group	Per cent composition of diet						Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl		Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	72	—	16	10	1	1	7.5 (1.3–24.8)	10	9	20.2	3.4±0.6	11.5±0.8	56.0±3.1
B	62	10	16	10	1	1	8.8 (2.7–25.5)	10	9	21.2	5.0±0.4	14.0±0.8	53.5±3.3
A′	72	—	16	10	1	1	7.5 (1.3–24.8)	18	12	23.5	6.5±1.0	23.5±1.6	36.7±4.7
B′	62	10	16	10	1	1	8.8 (2.7–25.5)	13	11	21.5	5.2±0.5	25.9±1.9	43.0±0.9

Elimination of corn meal produced no decrease but in fact an increase in thyroid weight (left lobes of comparable mice fed Purina Fox chow weighed about 1 mg.). Histologically, the thyroid glands from mice of groups A, B and C showed evidence of intense activation, that is, irregular, densely cellular follicles with small lumina containing thin wisps of colloid, as well as general hyperemia. Intense radioautographic images were observed over the colloid remnants. (These histological features were observed in almost all animal groups discussed in this paper. Therefore the histology will no longer be mentioned below, unless special features make it necessary). Since the absence of corn meal from the Modified Remington Diet did not interfere with the development of goiter in mice, the action of this diet *cannot* be attributed to the presence of a positive goitrogenic factor in corn.

Liver (Table 2). Inclusion of Wilson Extracted Liver Residue in the Modified Remington Diet produced increases in thyroid weight which were significant at 52 days (Group A vs. B; $P = 0.02–0.05$), but no longer so at 97 days (Group A′ vs. B′). The radio-iodine uptakes by the thyroid glands of both age groups fed on diets with and without liver were not significantly different. The weak positive goitrogenic activity which may exist in liver had little influence of the effect of the whole diet, since large goiters were readily obtained in the absence of liver; therefore the effect of the Modified Remington Diet on the thyroid *cannot* be attributed to the positive goitrogenic factor present in liver.

Wheat Gluten (Table 3). When wheat gluten at a 16% level in the control diet (Group A) was replaced by either corn meal plus liver (Group B) or corn meal alone (Group C), there was no significant effect on thyroid weight or on radio-iodine uptake. The large size of the thyroid glands in animals not receiving wheat gluten shows that the goiter-producing ability of the Modified Remington Diet *cannot* be attributed to any factor in wheat gluten.

Yeast (Table 4). When Dried Brewer's Yeast (Group A) was replaced by

TABLE 3. ROLE OF *wheat gluten* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: 56 Days)

Group	Per cent composition of diet						Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl		Initial	Fina	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	*72*	—	*16*	10	1	1	11.9 (1.4–37.6)	12	12	22.3	4.3±0.7	6.9±1.3	46.2±3.1
B	*78*	*10*	—	10	1	1	13.2 (2.3–41.7)	12	12	20.4	2.1±0.6	6.9±1.4	41.4±3.4
C	*88*	—	—	10	1	1	14.5 (0.8–44.1)	12	11	20.5	2.4±0.9	4.0±0.8	42.9±1.9

an equivalent amount of liver (Group B) or corn meal (Group C), no significant effects were observed on the weight or radio-iodine uptake of the thyroid gland. Therefore, the production of goiter by the Modified Remington Diet *cannot* be attributed to a positive goitrogenic factor in yeast.

Calcium Carbonate (Table 5). When the control diet containing 1% calcium carbonate (Groups A and A′) was compared to diets deprived of this salt (Groups B and B′), there was a decrease in thyroid weight, which was significant only in Group B′. This was no longer significant when the weights of the thyroid based on body weight, that is the relative thyroid weights, were considered. Other signs of thyroid activation (radio-iodine uptake, histological picture of stimulation) were equally intense in all groups. When 10% calcium carbonate was given in the diet (Groups C), the thyroid glands were similar to those of the control animals of Group A. Therefore, the goitrogenic action of the Modified Remington Diet *cannot* be attributed to the presence of calcium carbonate.

Sodium Chloride (Tables 6 and 7). The first experiment on the role of sodium chloride in the production of goiter by the Modified Remington Diet included mice (Group B, Table 6) receiving no added sodium chloride while the controls were fed a diet containing 10% sodium chloride (Group A). Survival was poor in the latter group. Both groups were sacrificed at 61 days at which time the mean weights of the left thyroid lobes were 4.2

TABLE 4. ROLE OF *yeast* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: 66 Days)

Group	Per cent composition of diet						Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl		Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	*72*	—	16	*10*	1	1	3.6 (1.9–5.5)	16	15	24.1±0.6	8.2±0.6	13.9±1.1	44.5±2.7
B	*72*	*10*	16	—	1	1	4.5 (2.7–6.7)	16	14	22.1±0.6	6.2±0.6	16.9±1.2	46.5±1.9
C	*82*	—	16	—	1	1	2.5 (1.2–4.1)	16	15	22.7±1.0	6.7±1.0	12.4±0.8	38.6±1.9

TABLE 5. ROLE OF *calcium carbonate* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: Group A, B, C, 61 Days; Group A′, B′, 51 Days)

Group	Per cent composition of diet						Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl		Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	*72*	—	16	10	*1*	1	1.4 (0.8–1.6)	13	12	22.4	5.8±1.2	13.2±1.6	40.5±1.1
B	*73*	—	16	10	—	1	1.4 (0.8–2.6)	13	11	22.9	6.3±0.9	9.2±1.7	36.2±2.3
C	*63*	—	16	10	*10*	1	1.4 (0.8–2.6)	13	12	17.7	0.5±0.7	13.5±1.6	43.0±2.3
A′	*72*	—	16	10	*1*	1	10.0 (7.1–14.2)	17	10	21.3	6.9±1.7	16.0±0.9	34.5±0.6
B′	*73*	—	16	10	—	1	9.9 (7.0–14.2)	16	9	16.8	2.8±0.8	13.2±0.6	35.1±1.8

mg. (Group B) and 14.7 mg. (Group A), the difference between means being highly significant. Furthermore, the I^{131} uptake by the thyroid glands of Group A was higher than that of Group B ($P = 0.02$–0.05). Histologically, the thyroids of the mice receiving sodium chloride (Group A) displayed the usual picture of stimulation, but in the absence of sodium chloride (Group B), the glands were composed of smaller spherical follicles containing fewer cells and larger colloid accumulations, and, therefore, displayed less stimulation. From these findings, it was concluded that withdrawal of sodium chloride interfered with the formation of goiter in mice fed the Modified Remington Diet.

Since repetition of this work did not always give uniform results, it was suspected that the basic diet even without added sodium chloride might contain enough of this salt as a contaminant to allow thyroid enlargement to occur. Hence, sodium and chloride determinations were carried out on the various food components of the Modified Remington Diet. Wheat gluten was the only foodstuff containing large quantities of both sodium (0.06–0.08%) and chloride (1.5%). Yeast contained 0.03–0.13% sodium

TABLE 6. ROLE OF *sodium chloride* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: Group A, B, 61 Days; Group A′, B′, 51 Days)

Group	Per cent composition of diet						Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl		Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	*63*	—	16	10	1	*10*	5.3 (3.9–7.6)	13	6	21.4±0.5	3.4±1.1	14.7±2.3	46.2±3.6
B	*73*	—	16	10	1	—	6.0 (4.3–8.3)	13	13	20.3±1.3	3.5±1.5	4.2±1.6	35.3±2.9
A′	*88*	—	—	10	1	*1*	11.4 (8.0–16.3)	16	12	21.0	7.1±0.7	12.0±0.8	33.1±1.1
B′	*89*	—	—	10	1	—	11.5 (8.1–16.4)	16	12	21.5	7.5±0.5	2.3±0.3	28.9±2.3

TABLE 7. ROLE OF *salts* IN THE PRODUCTION OF GOITER BY THE MODIFIED REMINGTON DIET

(Experiment Duration: 53 Days)

Group	Per cent composition of diet						Iodine content of diet (μg./100 gm.)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Corn meal	Liver	Wheat gluten	Yeast	$CaCO_3$	NaCl		Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	*84.4*	—	—	10	*2.6*	*3*	12.1	14	12	19.9	4.0±0.3	11.4±1.0	44.4±2.1
B	*87*	—	—	10	—	*3*	12.4	13	8	18.4	2.7±0.6	12.4±1.2	46.1±1.9
C	*90*	—	—	10	—	—	12.7	13	11	17.9	2.2±0.6	4.4±1.0	38.7±3.0

but only 0.01% chloride, while corn meal contained no detectable sodium or chloride. The experiment was, therefore, repeated in the absence of wheat gluten.

Under these conditions (Table 6, Group A′ and B′) the mean thyroid weight of mice in Group B′ (no sodium chloride) was 2.3 mg., while that of mice receiving 1% sodium chloride (Group A′) was 12.0 mg. Body weight gains were identical in both groups. (Thus the mouse can grow on a remarkably low sodium chloride intake, roughly 60 μg. of sodium chloride per day, in sharp contrast to the rat, whose growth on the Remington Diet without added sodium chloride is greatly reduced; Remington, 1937).

In addition, a diet containing 3% sodium chloride and 2.6% calcium carbonate (Table 7, Group A) was compared to one devoid of the calcium salt (Group B) and to a third devoid of both (Group C). The results not only confirmed the fact that withdrawal of sodium chloride from the Modified Remington Diet led to a pronounced decrease in thyroid weight (compare Group C with Groups A and B), but also showed that the presence of sodium chloride allowed the development of goiter in the absence of calcium carbonate (Group B) and that the addition of calcium carbonate did not produce a further increase in the weight of the thyroid (Group A).

Conclusion

Thyroid enlargement consistently occurred in mice fed the Remington Diet itself or the same diet modified by the removal of corn meal (Table 1), wheat gluten (Table 3), or yeast (Table 4). Withdrawal of liver had no statistically significant effect in two experiments (Table 1, Group B vs. Group C; Table 2, Group B′ vs. Group A′) and had a small effect in one (Table 2, Group B vs. Group A); but in no case did removal of liver from the diet prevent the occurrence of goiter (Tables 4 to 8). Calcium carbonate withdrawal produced a small decrease in thyroid weight in one experiment (Table 5, Group A′ vs. Group B′), but the body weights of the animals were also reduced and when the thyroid weights were calculated relative to body weights, they did not differ from those of the controls. In two other experiments (Table 5, Groups A and C vs. Group B; Table 7, Group A vs. Group B), the omission of calcium carbonate from the diet had no

significant effect on the relative or absolute thyroid weight. These results showed that the effect of the Remington Diet on the thyroid gland is not due to a "positive goitrogenic factor" in corn meal, wheat gluten, yeast, liver, or calcium carbonate.

The only constituent of the Remington Diet that exerted an important influence on the production of goiter was sodium chloride. Omission of this salt (Table 6, Group A′ vs. Group B′) caused a pronounced decrease in the thyroid weight, while large goiters always resulted from inclusion of sodium chloride in the diet at levels of 1 per cent (Table 6, Group A). This effect of sodium chloride was independent of the calcium carbonate content of the diet (Table 7, Group A vs. Group B) and was consistent when wheat gluten (contaminated by sodium chloride) was omitted from the diet.

These results definitely established the fact that sodium chloride had an effect on goiter production and a preliminary report on the subject was published (Axelrad and Leblond, 1952).

Relationship between the effects of iodine and sodium chloride on the thyroid gland. The quantitative experiment (Table 8)

At the lowest level of iodine intake (4.1 μg. iodine/100 gm. of diet; subgroups A, B, C and D), the thyroid glands were significantly larger than in all other groups receiving iodine supplements (8.3 to 13.1 mg./left lobe as against 0.8 to 2.5 mg.). When the four subgroups receiving different amounts of sodium chloride were compared, thyroids were larger and radioiodine uptakes higher in those animals that had the higher salt intake

TABLE 8. QUANTITATIVE EXPERIMENT SHOWING THE RELATIONSHIP BETWEEN POTASSIUM IODIDE AND SODIUM CHLORIDE IN CONTROLLING THE THYROID GLAND

(Experiment Duration: 68 and 69 Days)

Sub-groups	Per cent composition of diet					Iodine content of diet (μg./100 gm.)*	Iodine intake (μg./ mouse/ day)	Number of animals		Body weight (gm.)		Left thyroid lobe	
	Wheat gluten†	Yeast	$CaCO_3$	NaCl	Glucose			Initial	Final	Final	Gain	Weight (mg.)	I^{131} uptake (% dose)
A	16	10	1	*0*	*73.0*	4.1± 1.4	0.13	7	4	26.4	2.8±0.6	8.33±0.69	45.47±3.81
B	16	10	1	*0.1*	*72.9*	4.1± 1.4	0.13	7	6	26.8	2.5±0.7	9.75±1.27	55.85±3.63
C	16	10	1	*1.0*	*72.0*	4.1± 1.4	0.13	6	6	27.5	2.1±0.7	10.98±1.41	56.99±4.18
D	16	10	1	*3.0*	*70.0*	4.1± 1.4	0.13	6	6	27.2	1.9±0.5	13.13±0.80	60.85±2.88
A′	16	10	1	*0*	*73.0*	12.2± 1.3	0.39	7	7	28.9	4.9±0.8	1.13±0.13	16.80±2.75
B′	16	10	1	*0.1*	*72.9*	12.2± 1.3	0.39	7	7	28.6	4.5±0.7	1.20±0.11	19.54±1.88
C′	16	10	1	*1.0*	*72.0*	12.2± 1.3	0.39	7	6	25.8	2.1±0.8	0.98±0.13	29.69±4.59
D′	16	10	1	*3.0*	*70.0*	12.2± 1.3	0.39	7	6	25.9	1.4±0.7	1.40±0.20	35.09±2.11
A″	16	10	1	*0*	*73.0*	32± 3.8	1.0	7	6	26.1	2.6±1.9	1.04±0.14	4.82±0.96
B″	16	10	1	*0.1*	*72.9*	32± 3.8	1.0	7	7	28.1	4.1±0.6	1.20±0.19	7.68±1.35
C″	16	10	1	*1.0*	*72.0*	32± 3.8	1.0	7	7	25.6	0.9±1.1	1.11±0.16	12.65±1.82
D″	16	10	1	*3.0*	*70.0*	32± 3.8	1.0	7	3	25.6	2.2±0.7	0.77±0.29	12.25±1.61
A‴	16	10	1	*0*	*73.0*	100±11.8	3.2	6	5	27.6	4.4±0.6	2.48±0.28	2.80±0.25
B‴	16	10	1	*0.1*	*72.9*	100±11.8	3.2	7	6	25.4	1.3±0.9	1.93±0.11	4.49±0.63
C‴	16	10	1	*1.0*	*72.0*	100±11.8	3.2	7	7	29.6	5.7±0.7	1.56±0.17	5.45±1.04
D‴	16	10	1	*3.0*	*70.0*	100±11.8	3.2	7	7	27.1	3.3±0.6	1.40±0.19	6.65±1.01

* Values obtained by the Dry Digestion Method.
† Washed to remove sodium chloride.

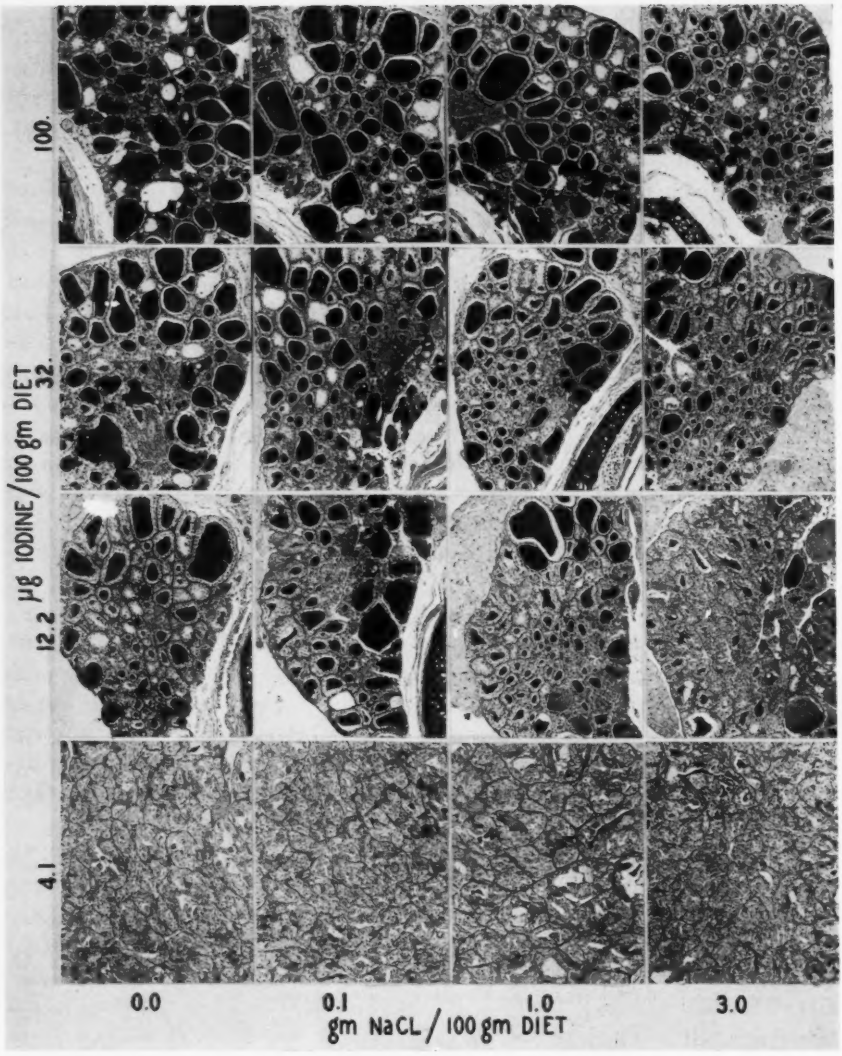

FIG. 1. Periodic acid-Schiff-hematoxylin stained thyroid sections from mice fed graded amounts of iodine and sodium chloride. (Quantitative Experiment) (Table 8). The 16 pictures represent typical thyroid glands from each one of the 16 subgroups in this experiment.

Each horizontal row of pictures corresponds to a certain iodine content of the diet, with the successive rows from bottom to top representing increasing iodine content.

Each vertical column of pictures corresponds to a certain level of sodium chloride content of the diet, with the successive columns from left to right representing increasing sodium chloride content.

The signs of activation (increase in cell size, decrease in the colloid content of the follicle) become more pronounced either as the iodine intake decreases or, except along the bottom row, as the sodium chloride intake increases.

(Table 8). Histologically, the glands of all animals on the low iodine intake showed the same characteristic signs of hyperactivity: intense hyperaemia, densely packed high follicular cells and almost complete disappearance of colloid (see Fig. 1, bottom row).

When the diets contained 12.2 μg. of iodine per 100 gm. of diet (*Subgroups A′, B′, C′ and D′*), the thyroid weights were small (0.98 to 1.4 mg.) and uninfluenced by sodium chloride intake, but the radio-iodine uptakes increased with increasing sodium chloride content of the diet (Table 8). Successive examination of thyroid histology in the 4 subgroups (Fig. 1, third row) showed that the signs of stimulation increased as the amount of sodium chloride in the diet increased, that is, the height of the cells became greater, the size of the colloid accumulations was decreased, their degree of stainability was reduced, their vacuolation was increased, and the vascularity of the gland was markedly augmented.

When the diets contained 32 μg. of iodine per 100 gm. of diet (*Subgroups A″, B″, C″ and D″*), the thyroids were of about the same weight as in the animals receiving 12.2 μg. of iodine/100 gm. of diet, but the radio-iodine uptakes were about one third as high as those at the previous iodine level. Examination of the 4 subgroups revealed that the radio-iodine uptake values increased (Table 8) and the histological picture showed greater activity (Fig. 1, second row) as the sodium chloride intake increased.

When the diets contained 100 μg. of iodine per 100 gm. of diet (*Subgroups A‴, B‴, C‴, and D‴*), there was a change in the pattern observed in the previous groups. On the one hand, the left thyroid lobe weights were *greater* (1.4 to 2.5 mg.) than at the previous iodine level. The differences between the thyroid weights of the two first corresponding subgroups (A″ vs. A‴ and B″ vs. B‴) at these two levels were significant. Furthermore, at this level of iodine intake, the thyroid weights *decreased* with increasing concentration of sodium chloride in the diet. On the other hand, the radio-iodine uptake values followed the trend set in the other groups since they were about half as great as the previous iodine level and increased with increasing sodium chloride intake. Histologically, the thyroids of animals receiving no added sodium chloride displayed flat to cuboidal follicular cells and large amounts of non-vacuolated, heavily stained colloid (Fig. 1, first picture of top row) which may have accounted for the weight increases recorded. The addition of sodium chloride to the diet at this iodine level still caused some histological changes in the thyroid (Fig. 1, top row, left to right). The number of cells appeared slightly greater, the capillaries somewhat fuller, the colloid was slightly decreased in amount and stainability, and a small degree of colloid vacuolation appeared.

Conclusion

The activation of the thyroid generally increased with increasing sodium chloride intake. It may also be emphasized that, when the 4 subgroups at

any one of the sodium chloride levels were compared, the activation of the thyroid always decreased with increasing iodine intake (compare the four pictures making up any column in Fig. 1).

At the lowest level of iodine intake (Table 8), the thyroids were smaller in the absence of sodium chloride (Subgroup A) than in its presence (Subgroups B–D), but in all cases they were goitrous as shown by weight, radio-iodine uptake and histological signs of intense activation. Thus, even in the absence of sodium chloride, a very low intake of iodine resulted in goiter.

DISCUSSION

Why does a low iodine intake cause goiter?

The only known role of iodide in thyroid physiology is as the raw material of which the thyroid gland synthesizes thyroid hormone. The amount of iodide required by mice for this synthesis can be calculated from the data of Hurst and Turner (1947, 1948). These authors suggested that, since the prevention of thiouracil goiter in mature male (Schwing) mice kept at 80° F. required 2.4 μg. D,L-thyroxine per 100 gm. body weight per day, this figure represents the daily output of hormone from the thyroid gland. The relative biological activities of D,L- and L-thyroxine allow us to calculate this daily output as 1.28 μg. of L-thyroxine, that is, 0.83 μg. of organic iodine, per 100 gm. body weight per day.

Assuming that under normal conditions the thyroid gland of the mouse is in a steady state with respect to iodine, as it is in the rat (Dougherty *et al.*, 1951), and that all the iodine entering the gland is utilized for thyroid hormone synthesis, it follows that the amount of iodine entering the gland must equal the amount of organic iodine that leaves as thyroid hormone.

Assuming further that the thyroids of the mature male mice used in the quantitative experiment had the same activity as those used by Hurst and Turner (1947, 1948), their minimum intake of iodine would have to be 0.83 μg. per 100 gm. body weight per day to maintain a normal thyroxine output from the thyroid gland.

The mature male mice of the quantitative experiment weighed approximately 25 gm. and consumed 3.16 gm. of food per day. It can be calculated that a diet supplying the minimum daily requirement for these animals (0.21 μg. iodine/day/mouse) would have to contain at least 6.6 μg. of iodine per 100 gm. of food. This value falls between those of the two groups on the lowest iodine intake in the quantitative experiment (in which the iodine estimations were thoroughly reliable). The animals fed a diet containing less than the minimum iodine requirement, that is, 4.1 μg. of iodine per 100 gm., developed goiter (Subgroup A, Table 8), while those fed diets containing 12.2 μg. of iodine or more per 100 gm. had thyroids of normal size (Subgroup A′.) The classical mechanism of iodine deficiency goiter may be postulated to explain these observations. That is, when the amount of

iodine available to the thyroid is not sufficient to cover the thyroxine requirement of the body, the resulting thyroid hormone deficiency induces an excessive secretion of thyrotrophic hormone, the thyroid enlarges and so becomes goitrous. Thus, the goiters observed in Subgroup A of the quantitative experiment may be considered as iodine deficiency goiters. Furthermore, since no sodium chloride was added to the diet of the animals of this subgroup, their goiters may be considered as uncomplicated iodine deficiency goiters.

Increase in iodine requirement due to sodium chloride

The goitrogenic action of inorganic salts has been investigated by several authors. Because of the claim that the soil of goiter regions was rich in calcium, the role of this element in goiter production has been investigated repeatedly but with contradictory results. The present work also showed no clearcut effect of calcium carbonate.

Hibbard (1933) reported hyperplasia and increase in weight of the thyroid in rats given 2% calcium chloride in their drinking water and fed on pearl barley, the iodine content of which was low. In order to determine which ion was responsible for the thyroid enlargement, Hibbard investigated the effects of calcium chloride, calcium lactate and sodium chloride. Calcium and sodium chloride caused hyperplasia. Calcium lactate had no effect. The author, therefore, concluded "that chlorine may act as a positive factor in the production of thyroid hyperplasia".

Remington (1937a) in his diet No. 342 studied the effect of sodium chloride on the thyroid by varying its amount from 0 to 4%. He found that rats deprived of this salt had thyroid glands with a smaller absolute weight and a higher iodine content. However, these rats grew poorly, and when their thyroid weights were expressed as grams per 100 gm. body weight, the differences in thyroid weight due to sodium chloride deprivation disappeared. Thus, in spite of the fact that withdrawal of the salt had caused the iodine content of the thyroid to increase threefold, Remington concluded that sodium chloride had no effect on the thyroid gland.

The present work definitely established that sodium chloride had an effect on goiter production (Tables 6 and 7). The mechanism of this effect was clarified in the experiment using different amounts of iodine and sodium chloride.

At the lowest level of iodine intake, the addition of sodium chloride caused a significant increase in thyroid weight and radio-iodine uptake (Table 8, Subgroup D vs. Subgroup A).

At the three other levels of iodine intake the addition of sodium chloride increased the radio-iodine uptakes but did not significantly alter the thyroid weights (Subgroups B′, C′, and D′ vs. Subgroups A′, etc.). At these levels, the histological appearance proved to be a sensitive indicator of the effect of sodium chloride. At each one of the three levels illustrated by

the upper three rows of pictures in Figure 1, there is from left to right an increase in activation of the thyroids (higher epithelium, less colloid, etc.), a fact indicating that, whatever the iodine intake was, the ingestion of sodium chloride increased the stimulation of the gland. The increase from the lowest to the highest level of sodium chloride, for a given iodine level, had approximately the same effect on the histological picture as decreasing the iodine intake from the said iodine level to the next lower level studied. Thus, the picture at the end of any one row was comparable to that at the beginning of the following row. Since the effects of reducing the iodine intake were similar to those produced by increasing the sodium chloride intake and since the effect of sodium chloride was observed at all iodine levels studied, it was felt that sodium chloride exerted its goitrogenic action by decreasing the amount of iodine available to the thyroid gland. This hypothesis has been fully supported by recent work in this laboratory in which sodium chloride was found to increase the loss of circulating iodide to the urine, thereby reducing the amount of iodide available to the thyroid (Isler *et al.*, in preparation).

Low iodine diets not producing goiter

Greenwald (1946) claimed that some low iodine diets did not produce goiter. A careful examination of such reported observations indicated that they could be classified into two categories: 1) those in which the iodine content of the diet had not been estimated (e.g. Hellwig, 1931) and 2) those containing casein (e.g. van Middlesworth, 1953). In a recent study in which purified casein low in iodine was included in a low iodine diet that otherwise caused massive thyroid enlargement, goiter did not develop (Axelrad and Leblond, 1954). The cause of the anti-goitrogenic effect of casein is not known. A factor in casein may compensate for the lack of iodine. Or perhaps the minute amounts of iodine retained during the commercial preparation of purified casein may be in an organic form (e.g. triiodothyronine) which is highly effective in inhibiting the secretion of thyrotrophic hormone from the hypophysis. In any case, casein-containing diets are so far the only known diets which may be low in iodine without causing goiter.

The iodine deficiency hypothesis

The present work demonstrates that the goiters observed in mice fed the Remington Diet are due to iodine deficiency, since 1) the diet had a low iodine content; 2) the occurrence of goiter could be prevented by addition of small doses of iodine to the diet; 3) none of the dietary components was essential for goiter production. The enhancement of goiter production by addition of sodium chloride can be completely explained by the fact that this salt makes a portion of the iodine entering the body unavailable

to the thyroid gland for hormone synthesis, a state of affair which enhances the degree of iodine deficiency already imposed by the low iodine content of the diet.

The effect of sodium chloride is basically different from that of the antithyroid substances (such as that described by Astwood, 1949), which impair the ability of the thyroid gland to synthesize its hormone. In contrast, the sodium chloride-containing Remington Diet maintains or enhances the functional ability of the thyroid gland, as shown by elevated radio-iodine uptakes (see tables) and rates of thyroid hormone secretion (Gross and Leblond, 1951). In fact, the results of the observations made in all animals at the lowest level of iodine intake (Table 8) suggest that, with or without sodium chloride, the iodine metabolism of the gland is accelerated under conditions of iodine deficiency. This conclusion is in keeping with the classical concepts of iodine deficiency. A low level of iodine intake would not allow the production of a sufficient amount of thyroid hormone; this would cause an excessive release of thyrotrophic hormone by the hypophysis; and as a result, the thyroid gland would enlarge. The enlarged gland would be potentially active. Given the raw material, iodine, it would actively produce thyroid hormone.

It may be concluded that the goiters observed in this work resulted from a failure of the thyroid gland to obtain an adequate supply of iodine.

SUMMARY

The Remington Diet, which consistently produces thyroid enlargement in rats and mice, was examined systematically in order to determine whether it contained any factor other than its deficiency in iodine that could be responsible for goiter production. The possible presence of a positive goitrogenic factor in any one of the foodstuffs used in this diet was investigated by comparing the weight, histology and function of the thyroid gland of mice fed the diet free of the foodstuff with those of mice fed the basic control diet.

Of all the foodstuffs composing the diet, only sodium chloride appeared to play an important role in the production of goiter (Table 6).

The relative roles of iodine deficiency and sodium chloride ingestion in goiter production were clarified by a study in which both the iodine and sodium chloride intake were systematically varied. At the lowest iodine level (4.1 μg. iodine per 100 gm. diet) goiter developed in the presence or absence of sodium chloride, but the weights and radio-iodine uptakes of the glands were increased by this salt. When the iodine intake was raised to 12.2 or more μg. of iodine per 100 gm. of diet, thyroid enlargement was prevented whether or not the animals received sodium chloride. Therefore, sodium chloride ingestion did not produce goiter in the absence of iodine deficiency. (This salt, however, still caused a pronounced activation of the thyroid gland, detectable by histological methods).

Thus none of the foodstuffs used in the Remington Diet, not even sodium chloride, was essential for the induction of goiter by a diet sufficiently low in iodine. The prevention of such goiters by a small dose of iodine completes the demonstration that iodine deficiency is the essential factor in their production.

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REFERENCES

ABBOTT, A. C.: *Canad. M. A. J.* **27:** 146. 1932.
ADOLPH, W. H. AND S. CHEN: *Chinese J. Physiol.* **4:** 437. 1930.
ADOLPH, W. H. and P. WHANG: *Chinese J. Physiol.* **6:** 345. 1932.
ASTWOOD, E. B.: *Ann. Int. Med.* **30:** 1087. 1949.
ASTWOOD, E. B., M. A. GREER AND M. C. ETTLINGER: *J. Biol. Chem.* **181:** 121. 1949.
AXELRAD, A. A. and C. P. LEBLOND: *Canad. M. A. J.* **67:** 675. 1952.
AXELRAD, A. A. and C. P. LEBLOND: *Canad. M. A. J.* **70:** 78. 1954.
BODNAR, J. AND J. STRAUB: *Biochem. Ztschr.* **227:** 237. 1930.
BOUSSINGAULT, J. B.: *Ann. Chim. Physiol.* **48:** 41. 1831.
BOUSSINGAULT, J. B.: *Ann. Chim. Physiol.* **54:** 163. 1833.
CHATIN, A.: *Compt. rend. Acad. d. sc.* **30:** 352. 1850.
CHATIN, A.: *Compt. rend. Acad. d. sc.* **31:** 280. 1850.
DOUGHERTY, J., J. GROSS AND C. P. LEBLOND: *Endocrinology* **48:** 700. 1951.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **139:** 371. 1923.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **142:** 263. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **152:** 116. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **152:** 128. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **152:** 132. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **152:** 135. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **152:** 141. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **152:** 153. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **142:** 246. 1924.
FELLENBERG, TH. VON: *Biochem. Ztschr.* **224:** 170. 1930.
GREENWALD, I.: *J. Clin. Endocrinol.* **6:** 708. 1946.
GROSS, J.: Thesis. Montreal. McGill University. 1949.
GROSS, J. AND C. P. LEBLOND: *Endocrinology* **48:** 714, 1951.
HARINGTON, C. R.: The thyroid gland, its chemistry and physiology, London, Oxford University Press, 1933.
HELLWIG, C. A.: *Arch. Path.* **11:** 709. 1931.
HERCUS, C. E., W. N. BENSON AND C. L. CARTER: *J. Hyg.* **24:** 321. 1925.
HERCUS, C. E. AND K. C. ROBERTS: *J. Hyg.* **26:** 49. 1927.
HIBBARD, J. S.: *Arch. Surg.* **26:** 648. 1933.
HURST, V. AND C. W. TURNER: *Am. J. Physiol.* **150:** 686. 1947.

HURST, V. AND C. W. TURNER: Univ. Missouri Coll. of Agric. Agric. Exp. Station Research Bull. 417. 1948.
JONES, D. W. C.: *Proc. Roy. Soc. Med.* **21**: 1217. 1928.
KIMBALL, O. P.: *Am. J. Pub. Health* **18**: 587. 1928a.
KIMBALL, O. P.: *J.A.M.A.* **91**: 454. 1928b.
KIMBALL, O. P. AND D. MARINE: *Arch. Int. Med.* **22**: 41. 1918.
KIMBALL, O. P., J. M. ROGOFF AND D. MARINE: *J.A.M.A.* **73**: 1873. 1919.
KLINGER, R.: *Schweiz. med. Wchnschr.* **51**: 12. 1921.
LUNDE, G.: *Biochem. Ztschr.* **193**: 94. 1928.
MARINE, D.: *J.A.M.A.* **104**: 2334. 1935.
MARINE, D. AND O. P. KIMBALL: *J. Lab. & Clin. Med.* **3**: 40. 1917.
MARINE, D. AND O. P. KIMBALL: *Arch. Int. Med.* **25**: 661. 1920.
MARINE, D. AND O. P. KIMBALL: *J.A.M.A.* **77**: 1068. 1921.
MARINE, D. AND C. H. LENHARDT: *Bull. Johns Hopkins Hosp.* **21**: 95. 1910.
MARINE, D. AND C. H. LENHARDT: *J. Exp. Med.* **13**: 455. 1911.
MAZZOCCO, P.: *Compt. rend. soc. biol.* **102**: 867. 1929.
MCCLENDON, J. F.: *J. Biol. Chem.* **55**: xvi. 1923.
MCCLENDON, J. F.: *Physiol. Rev.* **7**: 189. 1927.
MCCLENDON, J. F.: *J. Biol. Chem.* **102**: 91. 1933.
MCCLENDON, J. F. AND J. C. HATHAWAY: *J.A.M.A.* **82**: 1668. 1924.
MCCLENDON, J. F. AND A. WILLIAMS: *Proc. Soc. Exper. Biol. & Med.* **20**: 286. 1923a
MCCLENDON, J. F. AND A. WILLIAMS: *J.A.M.A.* **80**: 600. 1923b.
MIDDLESWORTH, L. VAN: *Proc. 19th Internat. Physiol. Congr.* p. 848, 1953.
MUGGIA, G.: Internat. Goitre Conference, Berne. 459. 1927.
NICOLAYSEN: Internat. Goitre Conference, Berne. 498. 1927.
PREVOST: 1830. Quoted by Harington, Loc. cit., p. 53.
REMINGTON, R. E.: *J. Nutrition* **11**: 343. 1936.
REMINGTON, R. E.: *Proc. Soc. Exper. Biol. & Med.* **37**: 652. 1937a.
REMINGTON, R. E.: *J. Nutrition* **13**: 223. 1937b.
SILBERSCHMIDT, W.: Internat. Goitre Conference, Berne. 1927.
STANBURY, J. B., G. L. BROWNELL, D. S. RIGGS, H. PERINETTI, E. DEL CASTILLO AND J. ITOIZ: *J. Clin. Endocrinol. & Metab.* **12**: 191. 1952.
WAGNER-JAUREGG, T.: Internat. Goitre Conference, Berne. 444. 1927.
WILSON, D. W., and E. G. BALL: *J. Biol. Chem.* **79**: 221. 1928.

THE EFFECT OF ANDROGENS ON FRUCTOSE PRODUCTION BY THE SEX ACCESSORIES OF MALE GUINEA PIGS AND RATS[1,2]

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IT HAS been established that the seminal vesicle is a primary site of origin of such constituents of the semen as fructose (Mann, 1946a; 1946b), citric acid (Humphrey and Mann, 1948; 1949), inositol (Mann, 1951), and ergothioneine (Mann and Leone, 1953). In the bull, ram, boar, and stallion, both fructose and citric acid are produced primarily by the seminal vesicle (Mann, 1949). In the rat, fructose is present in highest concentration in the dorsal prostate and coagulating gland, while citric acid is localized in the ventral prostate and seminal vesicle (Humphrey and Mann, 1948). The presence of androgen is necessary for the production of fructose (Mann and Parsons, 1947; Gassner and Hopwood, 1952) and citric acid (Humphrey and Mann, 1949) in the male reproductive system.

As part of a general program concerned with elucidation of hormonal regulation of the metabolism of the guinea pig seminal vesicle (Levey and Szego, 1955), a study was made of the influence of androgens upon the growth and fructose content of the sex accessories of juvenile rats and juvenile and adult guinea pigs. The results obtained are reported below.

MATERIALS AND METHODS

The animals used were juvenile rats of the Sprague-Dawley strain and juvenile and adult guinea pigs. All animals used in this investigation were bred in the departmental animal colony. Age and weight data are given in the appropriate section below. Androgens[4] were administered to orchidectomized guinea pigs subcutaneously as pellets or in oil solution. Hormonal treatment of rats is detailed in Table 4.

Tissues were removed from guinea pigs and rats under ether anesthesia. The secretions were retained with the tissues or analyzed separately (see text). The glands were homogenized, deproteinized with trichloroacetic acid, made to constant volume, and centrifuged for 10 minutes at 2000 r.p.m. Supernates were analyzed for fructose and other constituents as indicated.

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[4] The androgens used in this investigation were kindly furnished by Dr. Ernst Oppenheimer, through Ciba Pharmaceutical Products, Inc.

Yeast fermentation studies were carried out on several samples of secretion obtained directly from guinea pig seminal vesicles. These samples were homogenized in phosphate buffer (pH 7.0) and were centrifuged for 10 minutes at 2000 r.p.m. One cc. of buffered suspension of Fleischmann's yeast (washed free of yeast-non-fermentable, reducing contaminants) was added to aliquots of the supernates and the tubes were incubated for 1 hour at 35° C. The reaction was terminated by deproteinization with trichloroacetic acid, and the protein-free supernates were analyzed for fructose content and reducing activity. Control samples of seminal vesicle secretion were carried through the procedure without the yeast suspension. Triplicate determinations were in close agreement and average values were used to compile the data in Table 7.

Fructose was determined by the method of Roe (1934), reducing activity by the method of Folin and Malmros (1929), total carbohydrate by the method of Durham *et al.* (1950), pyruvic acid by the method of Bueding and Wortis (1940), citric acid by the method of Saffran and Denstedt (1948), and lactic acid by the method of Barker and Summerson (1941).

RESULTS

1. *Fructose in the seminal vesicle of the guinea pig*

Table 1 shows that fructose production in the seminal vesicle of the guinea pig was established at 20 days of age. The presence of visible secretion in this gland corresponded exactly with the appearance of fructose. In Table 2 it can be seen that practically all of the fructose was localized in the seminal vesicle secretion, with little remaining in the glandular tissue where it is presumably originally produced. Table 2 also demonstrates that while immature animals had smaller seminal vesicles, the concentration of fructose in the secretion, once established, was essentially at the adult level.

2. *Hormonal influence on fructose production in the sex accessories of immature guinea pigs and rats*

A group of normal, immature guinea pigs was injected with testosterone propionate in an attempt to hasten maturation of the seminal vesicles. The results, together with the protocol, appear in Table 3. It is clear that androgen stimulated the growth of the seminal vesicles but totally inhibited secretory activity and fructose production. The testes of the 28-day old animals injected with testosterone propionate were markedly atrophic as compared with those of the controls.

Similar studies were carried out in juvenile rats. The results are presented in Table 4. Testosterone propionate stimulated both the growth and fructose production of the sex accessories of immature rats. Gonadotrophin had a similar effect. In addition, there was no inhibition of either testis growth or sex accessory secretory activity in the androgen treated rats. High levels of fructose were present in the stimulated coagulating gland, dorsal prostate, and ampullary gland (median prostate). Only small amounts of this sugar could be detected in the seminal vesicle and ventral prostate of both injected and control animals. Hypertrophy of all the sex accessories was observed in the treated groups. The sex accessories of the

TABLE 1. EARLIEST APPEARANCE OF FRUCTOSE IN THE SEMINAL VESICLE OF THE GUINEA PIG

Age (days)	Body wt. (gm.)	Weight of testes (mg./100 gm. body wt.)	Weight of seminal vesicles (mg./100 gm. body wt.)	Weight of secretion (mg.)	Fructose in secretion (qualitative)[1]
14	174	138	36.8	0	0
16	151	156	27.8	0	0
16	190	139	35.8	0	0
16	209	136	38.3	0	0
16	217	136	46.1	0	0
16	233	174	60.9	0	0
18	265	189	60.4	15	+
20	298	197	70.5	20	+
21	263	—	35.7	20	+
23	300	193	70.0	0	0
23	307	203	84.4	75	++
23	308	181	76.0	20	+
23	320	175	53.8	20	+
23	347	207	66.9	0	0
25	288	—	—	—	++
25	412	—	—	—	+++
27	276	—	—	—	++
27	303	—	—	—	+++
28	308	201	51.9	80	+++
28	307	202	78.2	130	+++
29	370	193	92.4	150	+++
29	380	212	86.3	25	+
30	315	235	123.8	290	++++
31	425	260	73.9	52	++
31	417	237	86.3	210	++++
35	372	—	—	—	++++
35	397	—	78.6	200	++++
39	364	249	82.4	125	+++
39	395	246	96.7	223	++++
49	343	361	93.3	350	++++
49	356	309	78.7	210	++++
49	399	466	95.2	425	++++

[1] Fructose was estimated visually with + equivalent to a trace and ++++ representing an intense color reaction.

TABLE 2. FRUCTOSE IN SECRETION AND TISSUE OF THE GUINEA PIG SEMINAL VESICLE

	Body wt. (gm.)	Weight of seminal vesicles[1] (mg./100 gm. body weight)	Total weight of secretion (mg.)	Fructose in seminal vesicle tissue[2] (mg./100 gm.)	Fructose in seminal vesicle secretion (mg./100 gm.)
Immature	308	52	80	trace	225
	307	78	130	trace	100
	343	93	350	trace	157
	356	78	210	trace	211
	399	95	425	trace	123
Adult	809	167	1200	9	133
	919	187	1600	13	108
	755	242	1015	8	140
	945	138	2050	15	119

[1] Secretion-free.

[2] Tissues were rinsed several times in cold water in order to remove fructose adhering to the surface.

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES OF IMMATURE GUINEA PIGS

	Weight of testes (mg./100 gm. body weight)	Weight of seminal vesicles (mg./100 gm. body weight)	Weight of seminal vesicle secretion (mg.)	Fructose in combined seminal vesicle tissue plus secretion (mg./100 gm.)
Untreated (6)[1]	214 ± 18	78 ± 14	108 (25–210)	115 ± 30
Treated (5)[2]	76 ± 9	109 ± 9	0	0

[1] Animals were 28–31 days old at sacrifice.
[2] Starting on the day after birth, testosterone propionate was administered daily in subcutaneous injections of 1.0 mg. in 0.2 cc. of sesame oil. Animals were sacrificed on the afternoon following the final injection at 28 days of age.

TABLE 4. THE EFFECTS OF TESTOSTERONE PROPIONATE AND GONADOTROPHIC HORMONE ON THE SEX ACCESSORIES OF IMMATURE MALE RATS

Treatment	No. of rats	Body weight[1] (gm.)	Organ weights[1] (mg./100 gm. body weight)							Fructose in sex accessory tissue and secretion[1] (mg./100 gm.)				
			Testes	Adrenals	Seminal vesicles	Coagulating glands	Dorsal prostate	Ventral prostate	Ampullary glands	Seminal vesicles	Coagulating glands	Dorsal prostate	Ventral prostate	Ampullary glands
Untreated[2]	6	103 ± 3	523 ± 144	22.6 ± 1.5	33 ± 12	11 ± 2	40 ± 5	79 ± 13	9 ± 1	3 ± 1	4 (0–8)	14 ± 3	2.7 (0–5)	0
Testosterone propionate[3]	6	109 ± 7	725 ± 42	16.6 ± 2.3	831 ± 216	119 ± 21	200 ± 51	307 ± 39	46 ± 7	8 ± 3	60 ± 23	36 ± 10	4.8 (2–10)	67 ± 31
Gonadotrophic hormone[4]	7	78 ± 8	2070 ± 194	22.5 ± 2.3	870 ± 157	122 ± 13	253 ± 28	420 ± 51	42 ± 9	5 ± 2	80 ± 11	24 ± 5	1.5 (1–3)	42 ± 22
Castrate[5]	4	124 ± 3	—	25.4 ± 2.8	5 ± 1	2 ± 0	6 ± 0	6 ± 0	<1	0	trace	trace	0	0
Castrate[5] plus testosterone propionate[6]	2	105 (96, 114)	—	19.6 (17.1, 22.1)	583 (403, 763)	131 (125, 137)	213 (198, 228)	423 (400, 446)	50 (44, 56)	8 (7, 9)	71 (64, 78)	42 (40, 44)	5.5 (4, 7)	68 (55, 81)

[1] Mean ± standard deviation. The range is given in parentheses in cases in which the values were too low or observations too few to permit calculation of the standard deviation.
[2] Subcutaneous injection of 0.1 cc. Wesson oil daily for 24 days up to day of sacrifice at 37 days of age.
[3] Subcutaneous injection of 1.0 mg. testosterone propionate in 0.1 cc. Wesson oil daily for 24 days up to day of sacrifice at 37 days of age.
[4] Intraperitoneal injection of 1.0 unit gonadotrophic hormone (Upjohn's Gonadogen) daily for 24 days up to day of sacrifice at 37 days of age.
[5] Rats were castrated at 21 days of age and sacrificed at 41 days.
[6] Subcutaneous injection of 1.0 mg. testosterone propionate in 0.1 cc. Wesson oil daily for 20 days from day of operation to day of sacrifice.

castrate control animals were strikingly smaller than those of the intact controls, indicating that at 37–41 days of age there is some maintenance of these structures by the juvenile testis. Following the course of androgen treatment, the sex accessories of the castrate rats were comparable in size and fructose content to those of the similarly treated intact animals. It was of interest to note that the adrenal glands of the androgen injected intact group were reduced in size, an observation which is in accord with the majority of previous reports (cf. Parkes, 1945). Body weight was inhibited in the gonadotrophin injected animals.

3. *Effect of androgens on the fructose content of adult guinea pig seminal vesicles*

Castration caused a decline in the total fructose content of the seminal vesicles of adult guinea pigs (Table 5). Four different androgens (17-methyltestosterone, dehydroisoandrosterone, androsterone, and testosterone propionate), administered as subcutaneous pellets, were equally effective in maintaining the fructose level of the seminal vesicles of the castrated animals.

Since the pellet technique for administering hormone does not permit accurate estimation of actual dosage, an additional experiment was carried out in which testosterone propionate was administered to castrates at dosages of 0.5 and 2.0 mg. daily for 14 days prior to the day of sacrifice. The lower dosage of testosterone propionate was sufficient to restore to normal the fructose content of the castrate seminal vesicles. The weight of the seminal vesicle tissue and secretion showed the expected involutionary and restorative effects of castration and androgen therapy.

4. *Further studies on guinea pig seminal vesicle secretion*

As seen in Table 6, fructose accounted for less than half of the total carbohydrate present in guinea pig seminal vesicle secretion. Citric acid was also present in high concentration while lactate and pyruvate were absent. In the castrate, the levels of total carbohydrate, fructose, and citric acid were considerably reduced. In order to elucidate the nature of the non-fructose carbohydrate, yeast fermentation studies were carried out on two pooled samples of seminal vesicle secretion, obtained directly from the glands of several untreated adult guinea pigs. It is evident from Table 7 that fructose accounted for only about half of the total yeast-fermentable reducing activity present in the secretion. The possibility that the yeast-fermentable, nonfructose reducing material might be glucose has not been ruled out. In addition, there occurred a yeast-non-fermentable residue of unknown composition which accounted for about a third of the total reducing activity of the secretion.

TABLE 5. EFFECT OF ANDROGENS ON THE FRUCTOSE CONTENT OF THE SEMINAL VESICLES OF CASTRATED ADULT GUINEA PIGS[1]

Treatment	Quantity of androgen	Total fructose in seminal vesicle and secretion (mg.)[2]
Unoperated controls (8)	—	2.10 ± 0.69
21 day castrate controls (6)	—	0.25 ± 0.24
Androgens administered as accurately weighed subcutaneous pellets implanted on day of castration[3]		
	Mg. absorbed per day per kg. body weight	
Castrate + 17-methyltestosterone	0.79	2.20
	0.93	2.75
Castrate + dehydroisoandrosterone	0.47	1.30
	0.77	2.15
	0.78	2.40
Castrate + androsterone	0.50	1.55
	0.43	1.65
Castrate + testosterone propionate	0.86	2.20
	0.50	1.25
Androgen administered to 21 day castrate by subcutaneous injection in 0.2 cc. of sesame oil daily for 14 days		
	Mg. injected per day per animal	
Castrate + testosterone propionate	0.5	1.31
	0.5	1.54
	0.5	2.38
	0.5	3.12
Castrate + testosterone propionate	2.0	3.08
	2.0	5.32
	2.0	2.66

[1] Animals weighed 800–1000 grams.
[2] Standard deviations given for control groups.
[3] On day of sacrifice (45 days after implantation) pellets were removed, dried, weighed, and the average daily dose of hormone calculated for each animal.

TABLE 6. CONCENTRATIONS OF VARIOUS CONSTITUENTS OF INTACT AND CASTRATED GUINEA PIG SEMINAL VESICLE SECRETION[1]

Treatment of animals	Total carbohydrate	Fructose	Citric acid	Lactic acid	Pyruvic acid
Intact	328	145	179	5	0
	228	123	240	4	0
	370	157	288	0	0
	340	138	264	0	0
Castrate (21 days)	174	40	124	0	0
	130	41	154	0	0

[1] All values are expressed as mg./100 gm. secretion.

TABLE 7. YEAST FERMENTATION STUDIES ON GUINEA PIG SEMINAL VESICLE SECRETION

Sample	Weight of secretion (gm.)	Total reducing activity in control tube (A)	Yeast-ncn-fermentable reducing residue (B)	Yeast-fermentable reducing activity (A minus B)	Fructose in control tube (C)	Yeast-fermentable, non-fructose reducing activity (A minus B minus C)
1	3.900	250.3	82.8	167.5	72.3	95.2
2	5.083	235.1	70.2	164.9	84.6	80.3

All values are expressed as mg. fructose or fructose equivalents per 100 gm. secretion.

DISCUSSION

Sayles (1939) has reported that sperm are first produced in the guinea pig testis at 50 days of age. It has been demonstrated in the present study that fructose production in the seminal vesicle of this animal takes place at 20 days. Thus, the seminal vesicle must respond to the endogenous stimulus of the male hormone before maturation of the tubular elements of the testis occurs. The findings herein reported are in accord with those of Mann *et al.* (1951), who found that fructose production in the seminal vesicles of bull calves took place before the onset of spermatogenesis. The present studies on the rat indicate that fructose is produced as early as 37 days but observations were not made on younger animals. However, Porter and Melampy (1952) reported negligible secretion in the seminal vesicles of rats weighing 94 gm., and presumably at least 30 days old. Sperm heads appear in the rat testis at 33–35 days of age (cf., Moore, 1936). It is evident, therefore, that the time difference between the initiation of spermatogenesis and sex accessory secretory activity is not as pronounced in the rat as it is in the guinea pig.

The observation has been made that androgen suppressed secretory activity and fructose production in juvenile guinea pig seminal vesicles (Table 3), whereas in the castrated adult guinea pig, androgens were capable of replacing testicular function completely in maintaining the size and fructose level of the seminal vesicle. The failure of androgen to promote seminal vesicle secretion in immature guinea pigs may be related to the following observation. It was noted in histological preparations of the seminal vesicles of these animals (as opposed to similarly treated rats), that epithelial hyperplasia caused total occlusion of the glandular lumen, conceivably resulting in physical suppression of active secretion. The lack of androgenic inhibition of secretory activity of the sex accessories of immature rats suggests possible differences in the hormonal sensitivity of the secretory epithelium of the sex accessories of rats and guinea pigs.

The observation that the earliest appearance of fructose in the seminal vesicles of immature guinea pigs corresponded exactly with the presence

of visible secretion suggests that the secretory phenomenon is a unified physiological activity following an all or none principle with regard to the individual components of the secretion. Further support for this concept comes from the observation that the fructose concentration does not build up gradually as the animal matures, but is established at the adult level in the first days of activity of the immature gland. Whether this is the case with the other substances which are present in the seminal vesicle secretion has not been established.

The absence of significant quantitles of fructose from seminal vesicle tissue, and its concentration in the secretion, points to an energy-requiring, active transport mechanism for conducting fructose against an extreme concentration gradient. It seems likely that the regressive changes following castration are due, in part, to a failure of the energy-yielding mechanisms which maintain the normal permeability of the seminal vesicle. Additional studies in this laboratory (Levey and Szego, 1955) have, in fact, indicated that one of the primary changes observed in the secretory epithelium of the guinea pig seminal vesicle following castration is a selective decline in the aerobic phase of carbohydrate metabolism.

SUMMARY

The influence of androgen on the growth and fructose content of the male sex accessories has been studied in the rat and guinea pig. It was observed that in the untreated immature guinea pig, the time of initiation of secretion coincided with the first appearance of fructose in the seminal vesicle. The concentration of fructose in the glands of the immature guinea pig, once established, was essentially at the adult level. The administration of testosterone propionate to immature male animals stimulated growth and inhibited secretory activity (including fructose production) in guinea pig seminal vesicles but stimulated both functions in the sex accessories of rats. Testosterone propionate, androsterone, dehydroisoandrosterone, and 17-methyl-testosterone, administered as subcutaneous pellets, were equally effective in supporting the weight and fructose production of the seminal vesicles of castrated adult guinea pigs.

Two unidentified reducing substances, one yeast-fermentable and the other yeast-non-fermentable, have been described as constituents of guinea pig seminal vesicle secretion, along with fructose and citric acid.

The possible nature of secretory regulation by androgenic hormone was discussed on the basis of these and related findings.

REFERENCES

BARKER, S. B. AND W. H. SUMMERSON: *J. Biol. Chem.* **138**: 535. 1941.

BUEDING, E. AND H. WORTIS: *J. Biol. Chem.* **133**: 585. 1940.

DURHAM, W. F., W. L. BLOOM, G. T. LEWIS AND E. E. MANDEL: *Publ. Health Rep.* **65**: 670. 1950.

FOLIN, O. AND H. MALMROS: *J. Biol. Chem.* **83**: 115. 1929.
GASSNER, F. X. AND M. L. HOPWOOD: *Proc. Soc. Exper. Biol. & Med.* **81**: 37. 1952.
HUMPHREY, G. F. AND T. MANN: *Nature* **161**: 352. 1948.
HUMPHREY, G. F. AND T. MANN: *Biochem. J.* **44**: 97. 1949.
LEVEY, H. A. AND C. M. SZEGO: Submitted for publication. 1955.
MANN, T.: *Nature* **157**: 79. 1946a.
MANN, T.: *Biochem. J.* **40**: 481. 1946b.
MANN, T.: *Advances in Enzymology* **9**: 329. Interscience Publishers, Inc., N. Y. 1949.
MANN, T.: *Nature* **168**: 1043. 1951.
MANN, T., D. V. DAVIES AND G. F. HUMPHREY: *J. Endocrinol.* **6**: 75. 1951.
MANN, T. AND E. LEONE: *Biochem. J.* **53**: 140. 1953.
MANN, T. AND U. PARSONS: *Nature* **160**: 294. 1947.
MOORE, C. R.: *Am. J. Anat.* **59**: 63. 1936.
PARKES, A. S.: *Physiol. Rev.* **25**: 203. 1945.
PORTER, J. C. AND R. M. MELAMPY: *Endocrinology* **51**: 412. 1952.
ROE, J. H.: *J. Biol. Chem.* **107**: 15. 1934.
SAFFRAN, M. AND O. F. DENSTEDT: *J. Biol. Chem.* **175**: 849. 1948.
SAYLES, E. D.: *Physiol. Zool.* **12**: 256. 1939.

THE RELATION OF ASCORBIC ACID TO ADRENOCORTICAL FUNCTION DURING COLD STRESS[1]

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INTRODUCTION

THERE has been published from many laboratories a large volume of work showing the role of the adrenocortical hormones in the adaptation to cold as well as other forms of stress. It is well known now that during stress there is a significant reduction of adrenal ascorbic acid and cholesterol (Sayers *et al.* 1948) In spite of the many and varied attacks on this very important problem, there is still no satisfactory explanation of the role of ascorbic acid in the function of adrenocortical hormones; nor has there been a tenable postulation as to what part adrenal ascorbic acid reduction at the onset of stress may play in the initiation of adrenocortical function during stress. Also unexplained is the extent to which electrolyte changes may determine the survival to stress; and whether these electrolyte changes maybe influenced by ascorbic acid in intact or adrenalectomized animals. We have previously called attention to transitory electrolyte changes in the blood following the administration of ascorbic acid to animals at room temperature (Booker *et al.* 1951).

The recovery of intact and adrenalectomized animals from sublethal exposures to cold stress and the relation of this recovery to electrolytes in blood and certain tissues of the body, as influenced by adrenocortical hormones and ascorbic acid separately or in combinations, have not been described. In the present work we shall present evidence (a) that ascorbic acid is greatly more effective in increasing the survival time of intact than of adrenalectomized animals; (b) that combinations of high doses of ascorbic acid and low doses of cortisone or the reverse combinations have different effects on intact and adrenalectomized mice, and (c) that adrenalectomized and intact mice, when stressed and allowed to recover, show different plasma potassium and sodium levels.

METHODS

1. The adrenolectomized animals were given 0.9% sodium chloride instead of water to drink, and were fed purina chow pellets. They were not allowed access to food, water

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or saline during the exposure to cold. Animals treated with cortisone alone were administered 1 mg. Animals treated with ascorbic acid were administered 10 mg. Animals treated with both cortisone and ascorbic acid were in two categories (1) low cortisone (0.1 mg.), high ascorbic acid (50 mg.), and (2) low ascorbic acid (2 mg.), high cortisone (5 mg.). The doses indicated were administered per 100 gm. body weight by the intraperitoneal route. All drugs were administered approximately sixty minutes before the animals were exposed to cold (42° F). Survival time in cold was determined on animals in each category.

2. In other experiments, the effect of a shorter period of stress, exposure to cold for one hour, as modified by various treatments, on plasma sodium and potassium was determined. Animals were placed in individual containers during their exposures. There were ten animals in each category. Intact-unstressed-untreated and adrenalectomized-unstressed-untreated animals constituted controls. Intact and adrenalectomized experimental groups were composed of animals stressed for one hour and sacrificed immediately, as well as those stressed and allowed to recover for one hour at which time they were sacrificed. Ascorbic acid treated intact and adrenalectomized animals were run in each of the above categories. The animals were sacrificed by decapatation and blood was collected for potassium and sodium determination using the Barclay flame photometer. Extreme care was taken to keep hemolysis at a negligible level. The blood from each pair of animals in every category was pooled, making five samples for each group of ten animals on which sodium and potassium were determined.

RESULTS

It can be seen in Figure 1 that intact animals, treated with ascorbic acid, show much greater percentage survival than the untreated controls; and while the percentage survival of adrenalectomized mice treated with ascorbic acid is also higher than the untreated controls during the first through the fourth hour, the difference becomes less significant at the sixth hour. It is of interest that at the sixth hour there is a 60% survival of intact mice treated with ascorbic acid, while the adrenalectomized mice treated with ascorbic acid show no survival at all. Intact mice which were not treated with ascorbic acid show only a 5% survival. Figure 2 shows how closely the survival of adrenalectomized animals, treated with cortisone, parallels that of intact animals for five hours. When compared with the survival of adrenalectomized untreated mice (Fig. 1), it will be noted that more than a 40% difference exists between the cortisone-treated and the untreated group.

Figure 3 shows the results of combining cortisone with ascorbic acid. It will be noted that low doses of ascorbic acid plus high doses of cortisone are not as effective in increasing the survival time of adrenalectomized mice as in intact mice. In the latter group of animals, on the other hand, low doses of cortisone plus high doses of ascorbic acid are more effective than in adrenalectomized animals.

Table 1 shows the values of plasma potassium in the intact and adrenalectomized mice. Values are presented for the unstressed, the stressed and the recovered groups; also values are presented for groups of intact and adrenalectomized animals that were treated with ascorbic acid. It is note-

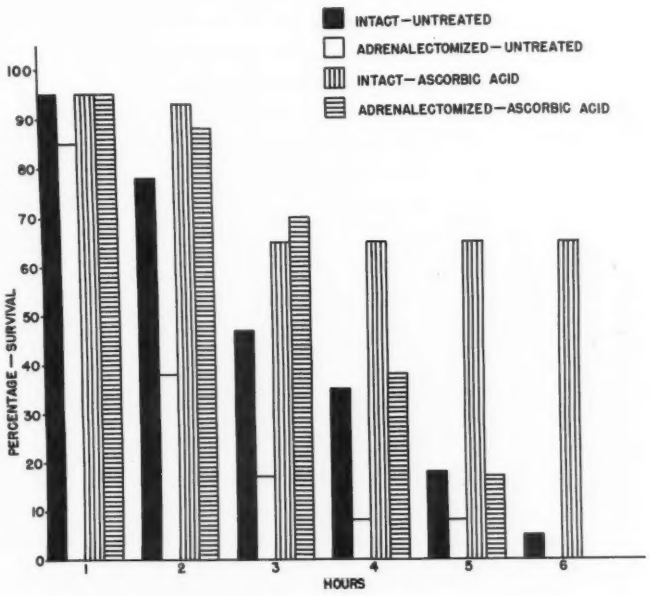

FIG. 1. The effects of ascorbic acid on the survival of intact and adrenalectomized mice. There were 80 animals in the group. Statistical analysis at third hour: Intact against adrenalectomized (untreated) P= <0.01; adrenalectomized untreated against adrenalectomized-treated, P=0.01 intact untreated against adrenalectomized treated, P=0.01.

TABLE 1. PLASMA POTASSIUM OF INTACT AND ADRENALECTOMIZED MICE, UNSTRESSED AND STRESSED, STRESSED AND ALLOWED TO RECOVER, AND ASCORBIC TREATED MICE

	No. animals	Mean plasma K (MEq/L)		P. value
Intact-Unstressed	10	5.14	±.07*	<0.001
Intact-Stressed	10	8.52	±.12*	
Adrenalectomized-Unstressed	10	6.04	±.12*	<0.001
Adrenalectomized-Stressed	10	13.10	±.16*	
Intact-Stressed-Recovery	10	5.64	±.13*	<0.001
Adrenalectomized-Stressed-Recovery	10	11.70	±.13*	
Intact-Stressed-Ascorbic Acid Treated	10	8.88	±.09*	<0.01
Intact-Stressed-Recovery Ascorbic Acid-Treated	10	5.10	±.05*	
Adrenalectomized-Stressed Ascorbic Acid-Treated	10	8.06	±.20*	<0.01
Adrenalectomized-Stressed-Recovery Ascorbic Acid-Treated	10	7.46	±.04*	

* Standard error of the mean.

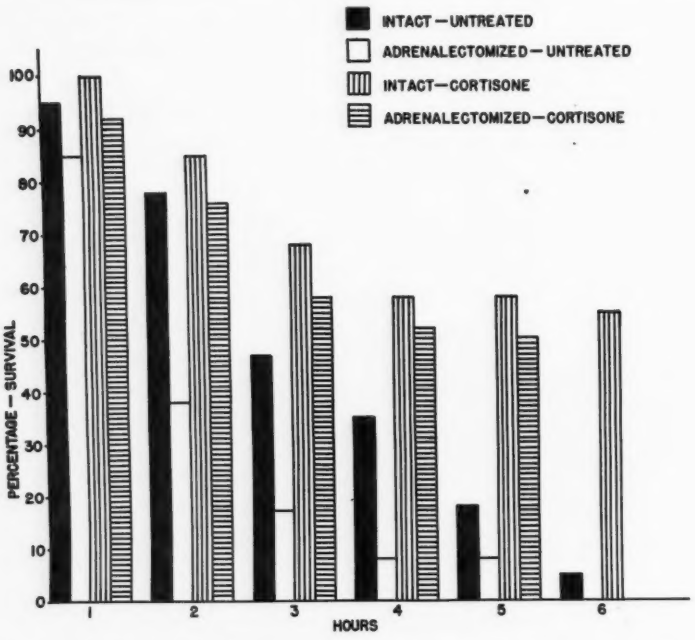

FIG. 2. The effects of cortisone on the survival of intact and adrenalectomized mice. There were 100 animals in the group. Statistical analysis at third hour: Intact untreated against intact cortisone treated, $P=0.01$; adrenalectomized untreated against adrenalectomized cortisone treated, $P=<0.01$.

worthy that in both the intact and adrenalectomized animals the potassium was elevated during stress; but in the intact groups, treated and untreated, there was no essential difference during the recovery period. On the contrary, the adrenalectomized animals showed a tremendous increase of plasma potassium during stress and even during the recovery period; but when treated with ascorbic acid the increase in plasma potassium was far less than that of the untreated groups in both stress and recovery periods.

There was no significant difference between the slightly elevated plasma sodium in the intact-untreated and intact-ascorbic acid treated groups stressed for one hour, although during the recovery the ascorbic acid treated groups showed 18% elevation of plasma sodium beyond the unstressed controls. It is of further interest that there was no significant difference at the end of stress between the plasma sodium of adrenalectomized-untreated animals and that of the adrenalectomized animals treated with ascorbic acid; but during recovery, neither the treated nor the untreated adrenalectomized animals showed plasma sodium values beyond the unstressed controls.

DISCUSSION

The effects of cold on the survival of untreated adrenalectomized mice are similar to the observations made by Zarrow (1942) in which six hours was the longest duration of survival reported. The results of our experiments permit the view that ascorbic acid is effective in increasing the survival time of animals when exposed to cold stress. The fact that ascorbic acid is more effective in intact than in adrenalectomized animals is of great interest, and strongly suggests that some functional relationship exists between adrenocortical hormones and ascorbic acid. That ascorbic acid is effective at all in increasing the survival of adrenalectomized animals, on the other hand, indicates that the vitamin has a limited sphere of action of its own. It may be speculated that ascorbic acid and adrenocortical hormones are probably peripheral in their functional relationship. Sherr (1952) has shown that the effects of cold on the survival time of adrenalectomized mice can be eliminated by cortisone; and he has further shown that the deleterious effects of high doses of cortisone seen at room temperature were not observed when animals were exposed to cold. It is of interest that relatively high doses of cortisone plus low doses of ascorbic acid were less effective in intact than in adrenalectomized animals where

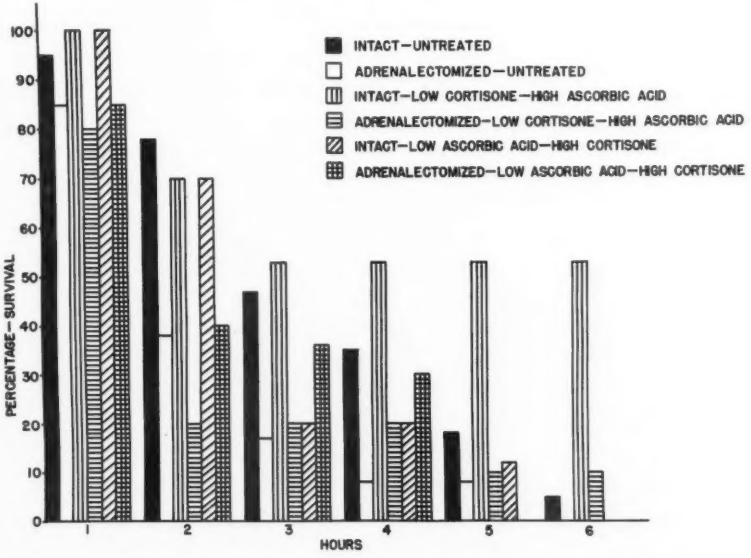

FIG. 3. The effects of combined dosages of ascorbic acid and cortisone on survival of intact and adrenalectomized mice. There were 40 animals in the groups. Statistical analysis at third hour: Low ascorbic acid high cortisone intact against adrenalectomized similarly treated ($P < 0.02$); high ascorbic acid low cortisone intact against adrenalectomized animals similarly treated, ($P < 0.02$).

high doses of cortisone and low doses of ascorbic acid were of greater effect. These observations stimulate the view that in the intact animals "diminishing returns" from cortisone administration can occur. It is noteworthy that in adrenalectomized animals, higher doses of cortisone can be tolerated and indeed are needed to raise the percentage survival. We have shown (Booker *et al.*, 1953) that adrenal homogenates, incubated with ascorbic acid and subsequently injected into adrenalectomized mice, increase the survival time beyond that of untreated controls and beyond the survival time of animals treated with cortisone or ascorbic acid individually. Shaffenberg, Masson and Corcoran (1950) believe that adrenal ascorbic acid is of great importance in the function of glucosteroids. Indeed they believe there is decreased function of the adrenal cortex in the absence of vitamin C.

In both the intact and the adrenalectomized animals during stress there was an elevation of the plasma potassium; but during recovery the intact animals showed no significant difference between the plasma potassium and that of unstressed intact controls. Adrenalectomized animals showed even a higher plasma potassium during recovery and during the stress period. This observation permits expression of the view that the application of cold stress to an animal may not result in the immediate release of adrenocortical hormones to control the electrolyte balance. A "lag" appears to exist in the release of adrenocortical hormones sufficient to control the plasma electrolytes. In this connection, we have been attracted by the work of Kuhel, Beck, Gershberg and Ralli (1954) who have observed that human subjects exposed to cold showed significant increases in plasma potassium the first and second hours of exposure.

The survival time of adrenalectomized-untreated animals in cold may be greatly influenced by their inability to handle potassium. We have observed in another group of experiments with rats that intact animals excrete more potassium in the urine both during stress and recovery periods than do adrenalectomized animals. We have shown also that sodium may be "mobilized" from certain tissues of the body, while the urinary excretion of sodium is diminished (Booker, *et al*, 1953). That ascorbic acid treated intact, as well as adrenalectomized animals to a more limited extent, handle their potassium better than the untreated animals, is of significance to us. This correlates well with the observation that ascorbic acid appears to increase the "life expectancy" of the animals exposed to cold. We believe, on the basis of the foregoing observations, that the handling of potassium appears highly important as a factor that may well determine the survival of animals during stress. On the other hand, we recognize that the mobilization and conservation of sodium during stress may well be an equal factor to be considered in the survival of animals during stress.

SUMMARY

Experiments have been described showing the effects of ascorbic acid, cortisone and combinations of these substances on survival of intact and adrenalectomized mice. Both cortisone and ascorbic acid were effective in increasing the survival of intact and of adrenalectomized mice subjected to cold stress. Ascorbic acid was more effective in intact animals than in adrenalectomized animals; but there was no appreciable difference between the effectiveness of cortisone in intact animals as against adrenalectomized animals when moderate doses were administered to both groups. Low doses of cortisone and high doses of ascorbic acid in combination were more effective in intact than in adrenalectomized animals. On the other hand, high doses of cortisone and low doses of ascorbic acid in combination were more effective in adrenalectomized than in intact animals. There is suggested a peripheral relationship between adrenocortical hormones and ascorbic acid. Adrenalectomized control animals during stress and recovery showed higher blood potassium levels than did comparable intact or ascorbic-acid-treated animals. The suggestion is made that ascorbic acid may facilitate the "handling" of potassium in the adrenalectomized animals subjected to cold stress.

Acknowledgment

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REFERENCES

Booker, W. M., R. S. Hayes, M. B. Sewell and F. M. Dent: *Am. J. Physiol.*, **166**: 374. 1951.

Booker, W. M., F. M. DaCosta, W. M. Jones and C. Froix: *Fed., Proc.*, **10**: 1. 1952.

Booker, W. M., F. M. DaCosta, W. M. Jones and C. Froix: *Fed., Proc.*, **12**: 1. 1953.

Kuhel, James, Edith Beck, Herbert Gershberg and Elaine Ralli: *Fed., Proc.*, **13**: 1. 1954.

Sayers, G., M. Sayers and C. N. H. Long: *Endocrinology*, **38**: 1. 1948.

Schaffenberg, C., G. M. Masson, A. C. Corcoran: *Proc. Soc., Exper., Biol., Med.*, **74**: 358. 1950.

Selye, H.: *Proc., Soc., Exper., Biol., Med.* **39**: 518. 1938.

Sherr, George: *Science*, **116**: 3025. 1952.

Zarrow, M.: *Proc. Soc., Exper., Biol., & Med.* **50**: 135. 1942.

THE INFLUENCE OF DIET ON THE RECOVERY OF THE RAT ADRENAL FROM STILBESTROL INDUCED STRESS[1,2]

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THE adrenal hypertrophy which follows the injection of estrogen is well documented. However, recovery of adrenal size and function following estrogen induced stress and the factors which modify the recovery phase have received little attention. Lynch (1952) observed that the adrenal continued to hypertrophy, in rats, for 3 weeks after discontinuing estradiol benzoate and that adrenal weight did not return to normal until 9 weeks after the cessation of treatment. The influence of nutrition on the recovery phase was not studied. Nevertheless, nutritional studies warrant consideration for chronic inanition alone serves as a stress in rats (Quimby, 1948), guinea pigs (D'Angelo *et al.*, 1948) and mice (Boutwell *et al.*, 1948). Furthermore, a high protein diet may increase the response of the pituitary-adrenal system to stress (Ingle *et al.*, 1947; Leathem, 1947; Hay *et al.* 1948; Moya *et al.*, 1948; Henriques *et al.*, 1949) and an adequate amount of protein is required for a maximal adrenal response to stress (Constantinides, 1950; Ershoff, 1952). It, therefore, was deemed of interest to investigate these hormone-nutritional interrelationships by comparing the adrenal response to, and recovery from diethylstilbestrol in adult rats fed purified diets with and without protein.

MATERIALS AND METHODS

Adult male rats of the Long-Evans strain were used when 250–350 gm. in weight. All stock animals were fed a diet consisting of Purina Fox Chow plus meat meal and supplemented weekly with lettuce and bread soaked in Mazola oil with vitamins A and D added.

Prior to actual experimentation the rats were fed an agar based 18% casein (Basal) diet (Table 1) for a 4-day period. Thereafter, one group of rats received 0.1 mg. (in oil) diethylstilbestrol (Stilbestrol) daily for 21 days and another group serving as nonin-

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TABLE 1. EXPERIMENTAL DIETS

Constituents	18% casein diet	Protein fere diet
	% dry weight	*% dry weight*
Casein	18.0	—
Sucrose	13.4	13.4
Glucose	20.2	38.2
Dextrin	18.2	18.2
Lard	24.2	24.2
Salt Mix (Wesson, 1932)	1.7	1.7
Cod Liver Oil	1.0	1.0
Agar	3.3	3.3

The agar is dissolved in an amount of water equal to 1.4 times the dry weight of the diet, i.e., 1400 gms. water plus 1000 gms. of dry diet. Equal to 2.4 kg. of wet diet. Vitamin Mixture Added:

Vitamin Supplements	*mgm./kg. wet diet*
Choline chloride	1000.0
Inositol	100.0
Riboflavin	3.2
Thiamin	2.0
Ascorbic acid	4.0
Pyridoxin hydrochloride	1.6
Calcium pantothenate	40.0
Niacin	40.0
p-Aminobenzoic acid	40.0
Alpha-tocopherol	40.0
2-Methyl-1,4 naphthoquinone	0.2
Biotin	0.2
Folic acid	0.2

jected controls was pair-fed against the stilbestrol injected rats. At the end of the injection period half the animals in each group were continued on the basal diet; the other half were fed a protein free diet (PFD), (Table 1). Adrenal glands were obtained at 0, 10, 20 and 30 days after the withdrawal of the hormone.

A second experiment was performed in which animals were fed a protein free diet (PFD), instead of an 18% casein diet, during the injection period. Post-injection adrenal morphology was observed in rats at the same intervals and under the same dietary conditions as in the first experiment.

The adrenal glands were fixed in a modified formalin solution (Baker *et al.*, 1948) and embedded in 10% gelatin (Zwemer, 1933). Sections were cut at 15 μ on a freezing microtome, stained and mounted in glycohrogel (Zwemer, 1933). Sudan IV was demonstrated by the use of Herxheimer's solution (Cowdry, 1948). This technique roughly measures the total neutral lipid but does not demonstrate masked fat (Baker *et al.*, 1948). Adrenal phospholipids and neutral fats were stained with sudan black B (Baker, 1944) and the presence of unsaturated glycerides was indicated by a positive reaction with the metachromatic stain, Nile Blue Sulphate (Lison, 1936). Cholesterol esters were tested by the Schultz modification (1924) of the Lieberman-Burchard technique.

RESULTS

Adrenal Weights: Stilbestrol increased the actual adrenal weight 110% in 20 days in animals fed the basal diet (Table 2) The effect was accentuated on an organ weight/body weight basis. A sharp reduction in adrenal weight followed hormone withdrawal but the glands of stilbestrol treated rats remained significantly heavier than those of their controls throughout

TABLE 2. INFLUENCE OF DIET ON POST-INJECTION CHANGES IN ADRENAL GLAND WEIGHT IN STILBESTROL INJECTED RATS

Recovery (days)	Group	Injection diet: basal			Injection diet: PFD		
		Body wgt. (gm.)	Adrenal weight		Body wgt. (gm.)	Adrenal weight	
			Actual wgt. (mg.)	Relative wgt. (mg./100 gm.)		Actual wgt. (mg.)	Relative wgt. (mg./100 gm.)
0	Inj.	243	71.6±6.8	32.6±3.6	221	60.9±6.9	27.4±1.9
	Cont.	250	33.6±1.8	13.4±1.5	266	34.1±0.8	13.1±0.8
10	Inj: Basal	297	42.6±2.9	14.0±1.0	320	42.8±5.5	13.6±0.6
	Cont: Basal	356	33.3±1.7	9.6±1.1	341	33.9±2.6	10.1±0.7
	Inj: PFD	235	35.3±3.1	16.0±1.6	211	48.2±5.7	23.5±3.7
	Cont: PFD	323	29.3±1.4	9.1±0.4	279	31.6±1.2	11.4±0.5
20	Inj: Basal	378	53.5±3.7	14.1±0.8	348	49.4±3.6	14.6±0.5
	Cont: Basal	378	40.4±2.9	10.6±0.5	396	38.1±2.2	9.7±0.6
	Inj: PFD	245	36.5±2.6	14.9±1.0	200	38.8±4.8	18.8±1.5
	Cont: PFD	270	28.1±1.5	10.6±0.8	271	29.1±1.6	10.8±0.4
30	Inj: Basal	402	41.3±2.5	10.3±0.5	396	46.4±1.0	11.9±0.6
	Cont: Basal	418	34.7±3.3	8.3±0.7	436	32.2±1.4	7.5±0.6
	Inj: PFD	230	36.8±2.4	16.4±1.6	214	29.9±3.0	14.0±1.0
	Cont: PFD	245	27.7±2.0	11.4±0.8	232	24.3±1.1	10.5±0.4

the course of recovery. If protein was withdrawn from the diet after the last hormone injection, adrenal atrophy was significantly greater on an actual weight basis than that which occurred when protein was fed. Adrenal weight relative to body weight, however, indicated that a protein free diet did not accentuate adrenal atrophy. In fact, the relative adrenal weight of rats fed protein were less than those of rats deprived of protein (Table 2,). The sharp increase in body weight during recovery on the basal diet following the cessation of injections without a similar response on the part of the adrenals necessarily reduced the relative adrenal weight (Table 2).

Stilbestrol treatment, concomitant with the feeding of a diet lacking protein resulted in a significant adrenal hypertrophy of approximately 78% (Table 2) which, however, was significantly less than when dietary protein was available during the injections (*vide supra*). Adrenal weight decreased during the post-injection phase regardless of diet. However, when 18% casein was fed, adrenal weight was still above control levels 30 days after injections. Feeding a 0% casein diet reduced adrenal weight below normal but adrenals from stilbestrol injected rats were larger than non-treated controls. The disparity of adrenal weights between rats fed protein and those continued on a protein free regime was essentially reversed by relating adrenal weight to body weight, the adrenals of rats fed PFD were relatively larger than the adrenal of rats fed casein (Table 2). The sharp increase in body weight during recovery on basal diet was not matched by a similar response in adrenal weight with the result that relative adrenal weight was reduced.

Adrenal Cholesterol: Schultz positive material in the normal adrenal cortex was concentrated in the Zona glomerulosa and the peripheral areas of the fasciculata, and became progressively less dense and uniform centrally. Injection of stilbestrol depleted the gland of Schultz positive material.

A return of adrenal cholesterol was observed, within 10 days post-injection, in rats fed on the basal diet. Increases in the size of the droplet and in the intensity, density and uniformity of distribution were noted with time. The stain reaction served to more sharply delineate the central and peripheral portions of the fasciculata at day 20 of recovery. Further increases in cholesterol, occurring throughout the entire cortex, were observed on day 30 of recovery. If, following stilbestrol, the rats were fed a protein free diet, Schultz positive material did not appear in the adrenal until 20 days after the cessation of treatment. At no time during the recovery phase was the adrenal cholesterol pattern of these animals characteristic of the normal. The feeding of PFD during both the injection and recovery periods delayed the appearance of a positive reaction until day 30 of recovery.

The absence of protein from the diet fed to non-injected animals caused no discernible alteration from the normal cholesterol pattern. The regime of pair-feeding during the treatment period produced the least noticeable change in the adrenal cholesterol of the control rats. This was immediately corrected in the recovery phase.

Neutral Fat and Phospholipid (sudan black B): Sudnophilic material, very heavily and uniformly distributed, was found concentrated in glomerulosa cell cytoplasm and in the peripheral area of the fasciculata. A relatively sharp decrease in intensity and uniformity of staining occurred in mid-fasciculata and continued through the reticularis. There was considerable degree of variation in this pattern, especially in the fasciculata; the very strong positive reaction sometimes continued to the medullary border (Fig. 1). Treatment with stilbestrol caused major changes in the distribution of sudanophilic lipids of the adrenal cortex. Except for some widely scattered lipid aggregations found only on the outer quarter of the fasciculata, the adrenal cortex was devoid of stainable material (Fig. 2). Regardless of diet, an immediate return to normal was noted upon cessation of injections (Figs. 3 and 4).

Neutral Fat (Herxheimer Stain): The pattern of adrenal cortical lipid in adrenals examined for the presence of sudan IV stainable fat was similar to that observed in the studies of adrenal cholesterol. Adrenals of stilbestrol injected animals were devoid of stainable material except for traces in the extreme peripheral quarter of the fasciculata.

An immediate return to normal was noted after the cessation of injections when protein was fed but a delay was evident if protein was withdrawn from the diet. Rats fed the basal diet during the injection period

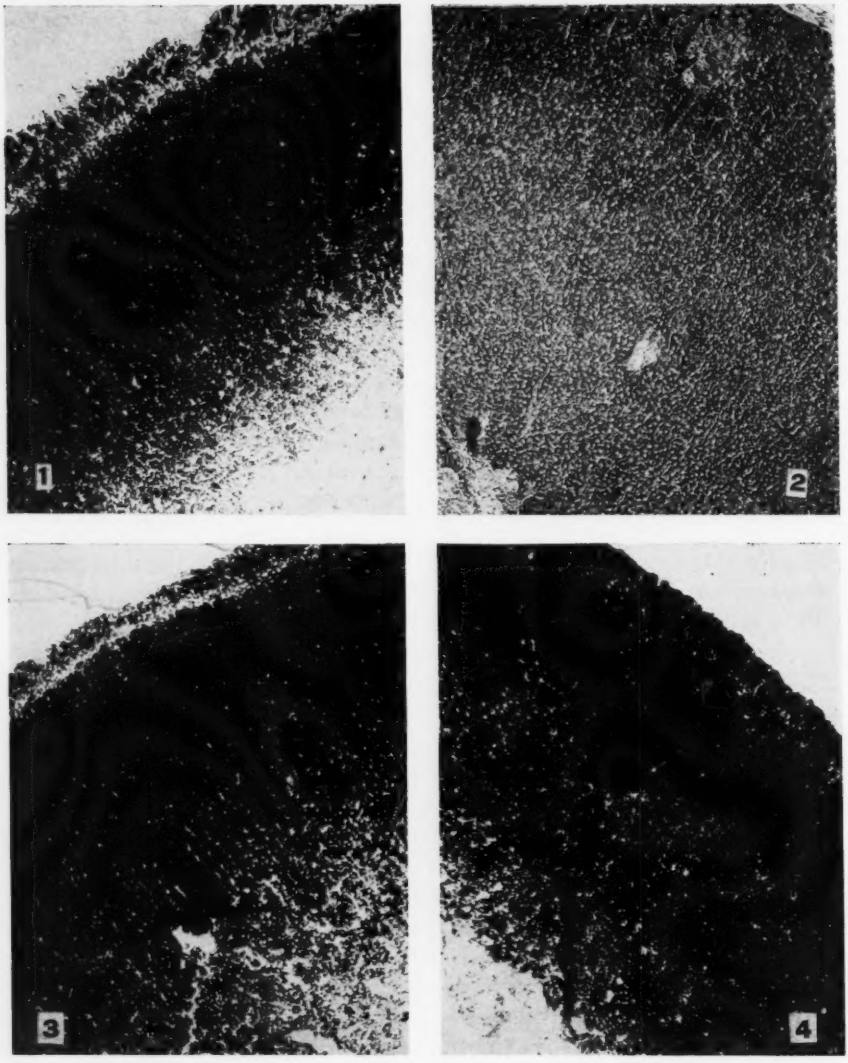

PLATE 1

Sections (15 μ) of the adrenal cortex of adult male rats. Stained with sudan black B. Magnification, 300×.

FIG. 1. Control group at "0" days recovery. Note characteristic histochemical pattern and presence of transition zone. Dietary variations are without effect.

FIG. 2. Stilbestrol injected group at "0" days recovery. Marked depletion of sudanophilia and transition zone is noted. Variation in diet is without influence.

FIG. 3. Stilbestrol injected group recovering on basal diet. *Injection diet: Basal.* Immediate reappearance of sudanophilia and transition zone (10 days). *Injection diet: PFD.* Immediate reappearance of normal sudan black B pattern. Transition zone does not reappear until day 30.

FIG. 4. Stilbestrol injected group recovering on PFD: *Injection diet: PFD.* Sudan black B reappears within 10 days of the cessation of injections. Transition zone does not reappear during the course of the experiment. *Injection diet: Basal.* Sudan black B is recouped within 10 days after treatment is stopped. Transition zone reappears by day 20 (not shown).

and fed PFD during the recovery phase exhibited a 10-day delay in adrenal lipid assimilation. A further 10-day delay was noted in those animals which received PFD throughout the entire experiment. Extracellular lipid aggregations were observed in all adrenals 30 days after injections were discontinued.

Unsaturated Glycerides (Nile Blue Sulphate): The control rat adrenal was devoid of unsaturated glycerides. Neither stilbestrol nor variation in diet altered the normal histochemistry.

Transition Zone: A sudanophobic "sublomerular" zone was present in the adrenals of normal male animals (Fig. 1). The injection of stilbestrol resulted in the disappearance of this zone (Fig. 2).

In the series of animals continued on the basal diet after the cessation of injections the sudanophobic area made its reappearance on day 10 of recovery whereas it did not reappear until day 30 in the adrenals of those animals transferred to PFD (Fig. 3). The absence of dietary protein, during the period of stilbestrol injections, retarded the reappearance of the sudanophobic area. The transition zone was not observed, in animals recovering on the basal diet after being fed PFD during the injection period, until day 20 of recovery and did not reappear at all in treated animals which received PFD throughout the injection and recovery periods.

DISCUSSION

Stilbestrol administration for 3 weeks induced marked adrenal cortical hypertrophy, a severe depletion of sudanophilic lipids and cholesterol and a loss of the transitional zone. The cortical hypertrophy was more pronounced than that obtained with a similar dose of estradiol benzoate injected for 3 weeks (Lynch, 1952). Furthermore, the cytochemical modifications of the adrenal in response to stilbestrol are far more extensive than those induced by physiological doses of estradiol benzoate (Greep and Jones, 1950). Cortical hypertrophy and altered cytochemistry may constitute presumptive evidence for increased secretory activity induced by stress (Popjack, 1944; Greep and Deane, 1949).

Stilbestrol imposed a caloric restriction which increased adrenal size in pair-fed control rats. Similar observations were recorded by Quimby (1948), D'Angelo *et al.* (1948) and Boutwell *et al.* (1948). However, cortical lipids and cholesterol were not altered by the caloric restriction of paired-feeding suggesting a gradual stress to which the gland has compensated (Sayers and Sayers, 1949).

The degree of cortical hypertrophy induced by stilbestrol was limited by feeding a protein free diet but nevertheless was significantly greater than control adrenal weights. Apparently a minimum level of dietary protein is necessary for an adequate adrenal response to stress (Constantinides, 1950) and may or may not be related to an impaired synthesis of ACTH by the hypophysis (Handler and Bernheim, 1950; Sayers *et al.*, 1950).

Rats treated with 0.1 mg. of estradiol benzoate for 3 weeks exhibited a continued increase in adrenal weight for 3 weeks after the cessation of injections. Normal adrenal weights were regained 9 weeks after withdrawing the hormone (Lynch, 1952). Substituting stilbestrol in a similar experimental design led to a more marked adrenal hypertrophy during the injection period and a precipitous decrease in adrenal weight within 10 days after stopping the hormone indicating that the two compounds are metabolised at different rates. The mode of recovery is complex but seems initially due to the cessation of stilbestrol injections, for caloric restriction is usually terminated before the injection period ends as indicated by a return of food intake to normal by day 15 of the injection period.

Recovery of sudanophilia and cholesterol in the adrenal cortex is clearly influenced by nutrition. An 18% casein diet permits the reappearance of sudanophilia and cholesterol in the cortex on day 10, but normal concentrations are not seen until 30 days after the last injection. Cortical sudanophilia does not appear until day 20 and is subnormal on day 30 when a 0% casein diet is fed. These latter animals seem not to have recovered fully from the injections of stilbestrol, and the absence of protein from the diet provides a source of continued stress.

The presence of a transition zone, which occurs in the normal rat adrenal, is thought to be indicative of a low level of cortical activity, whereas the converse is suggested by the absence of this zone (Greep and Jones, 1950). The zone disappears when stilbestrol is injected and returns on cessation of injections. Reappearance may be taken as an indication that the adrenal is recovering from the heightened secretory activity of the treatment period and is returning to a lower level of cortical activity at which the transition zone is normally present. Absence of dietary protein delays the reappearance of the transition zone for 20 days. Further delay in the reappearance of the transition zone is caused by feeding PFD during the injection period. The transition zone reappears by day 20 of recovery in treated rats transferred to basal diet but does not return at all, during the 30-day recovery period, in the adrenals of those rats continued on PFD. There is a general agreement between these data and the observations on the recouping of normal adrenal histochemistry and decrease in relative adrenal weight which would seem to indicate that the presence of this zone may constitute a fair index of the return to recovery by the previously stressed animal. However, the coincident occurrence of the transition zone and the slight but significant hypertrophy of the adrenals of PFD fed controls presents the question as to the relative significance of these morphological and histochemical factors as criteria of recovery.

SUMMARY

Stilbestrol induces a marked adrenal hypertrophy, depletion of sudanophilic and Schultz reactive material and the disappearance of the transition

zone in adult male rats fed a diet containing 18% casein. The absence of protein from the diet fed during the injection period produced no further changes in adrenal histochemistry but did decrease the degree of adrenal hypertrophy.

Recovery on the basal diet after the cessation of injections allows the immediate return of adrenal phospholipid and a more gradual reappearance of neutral fat, Schultz reactive material and transition zone. There is a decrease in relative adrenal weight to near control levels but the adrenal of the treated animal remains significantly heavier than the control glands. The absence of protein from the recovery diet markedly inhibits the recovery of the adrenal on the basis of the criteria observed here.

Feeding a protein deficient diet concomitantly with the injection of stilbestrol allows a further 20-day period of protein depletion. Such a regime produces a pronounced delay in the recouping of the normal histochemistry, morphology and weight relationships of groups recovering on either the basal or PFD diets beyond the times noted for similar groups which had received a diet containing adequate protein during the injection period.

Caloric restriction imposed by pair-feeding against stilbestrol injected rats produces a slight but significant hypertrophy of the adrenal of control animals. There is a return to normal weight relationships during the recovery period except in those pair-fed control groups recovering on PFD. The adrenals of these rats are significantly hypertrophied. No alteration from the normal histochemistry and morphology can be demonstrated in control groups.

REFERENCES

Baker, B. L., D. J. Ingle, C. H. Li and H. M. Evans: *Am. J. Anat.* **82:** 75. 1948.

Baker, J. R.: *Quart. J. Micro. Sci.* **85:** 1. 1944.

Boutwell, R. K., M. K. Brush and H. P. Rusch: *Am. J. Physiol.* **154:** 517. 1948.

Constantinides, P.: *Fed. Proc.* **9:** 25. 1950.

Cowdry, F. V.: Laboratory Technique in Biology and Medicine, 2nd Edition, Williams and Wilkins, Baltimore, Md. 1948.

D'Angelo, S. A., A. S. Gordon and H. A. Charipper: *Endocrinology* **42:** 399. 1948.

Deane, H. W. and R. O. Greep: *Am. J. Anat.* **79:** 117. 1946.

Deane, H. W. and J. M. McKibbin: *Endocrinology* **38:** 385. 1946.

Erahoff, B. H.: *Vitamins and Hormones* **10:** 79. 1952.

Greep, R. O. and H. W. Deane: *Annals N. Y. Acad. Sci.* **50:** Art. 6; 596. 1949.

Greep, R. O. and I. C. Jones: *Rec. Prog. in Hormone Research* **5:** 197. 1950.

Handler, P. and F. Bernheim: *Am. J. Physiol.* **162:** 368. 1950.

Hay. E. C., J. L. Prado and H. Selye: *Can. J. Research* **E26:** 212. 1948.

Henriques, S. B., O. B. Henriques and H. Selye: *Endocrinology* **45:** 153. 1949.

Ingle, D. J.: *Am. J. Physiol.* **124:** 369. 1938a.

Ingle, D. J.: *Am. J. Physiol.* **124:** 627. 1938b.

Ingle, D. J.: *Endocrinology* **24:** 194. 1939.

Ingle, D. J., M. C. Prestrud, C. H. Li and H. M. Evans: *Endocrinology* **41:** 170. 1947.

Leathem, J. H.: *Proc. Soc. Exp. Biol. and Med.* **64:** 90. 1947.

LISON, L.: *Histochemie Animale*, Gauthier-Villars, Paris. 1936.
LYNCH, K. M., JR.: *Annals N. Y. Acad. Sci.* **55:** Art. 4; 734. 1952.
MOYA, F., J. L. PRADO, R. RODRIGUEZ, K. SAVARD AND H. SELYE: *Endocrinology* **223.** 1948.
POPJACK, G.: *J. Path. Bact.* **56:** 485. 1944.
QUIMBY, F. H.: *Endocrinology* **42:** 263. 1948.
SAYERS, G.: *J. Clin. Endocrinology* **9:** 656. 1950.
SAYERS, G., M. A. SAYERS, E. G. FRY, A. WHITE AND C. N. H. LONG: *Yale J. Biol. Med.* **16:** 361. 1944.
SAYERS, G., M. A. SAYERS, T. Y. LIANG AND C. N. H. LONG: *Endocrinology* **38:** 1. 1946.
SAYERS, G. AND M. A. SAYERS: *Annals N. Y. Acad. Sci.* **50:** Art. 6; 522. 1949.
SAYERS, G., C. P. CHENG AND L. T. SAMUELS: cited by L. T. SAMUELS in *Progress in Clinical Endocrinology*, Grune and Stratton, New York. 1950.
SCHULTZ, A.: *Centralbl. f. Allg. Path.* **35:** 314. 1924.
SELYE, H.: *Endocrinology* **21:** 169. 1937.
SIMPSON, M. E., H. M. EVANS AND C. H. LI: *Endocrinology* **33:** 261. 1943.
TEPPERMAN, J., F. L. ENGEL AND C. N. H. LONG: *Endocrinology* **32:** 373. 1943.
ZWEMER, R. L.: *Anat. Rec.* **57:** 41. 1933.

THE EFFECT OF HYPOPHYSECTOMY, CRYPTORCHIDISM, ESTROGEN AND ANDROGEN UPON THE LEVEL OF HYALURONIDASE IN THE RAT TESTIS[1]

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THE presence of a "spreading factor" in testes of rats, rabbits and guinea pigs was first observed by Duran-Reynals (1928, 1929). Chain and Duthie (1939, 1940) demonstrated that the "spreading factor" is identical with an enzyme isolated from autolysates of Type II pneumococci by Meyer, Dubos and Smith (1937), and designated by the latter authors as hyaluronidase in view of its capacity to depolymerize hyaluronic acid. Subsequently the concentration of hyaluronidase in testes of rats placed under various experimental conditions has been studied by several investigators.

Sprunt *et al.* (1939) noted a decrease of hyaluronidase concentration in rat testes after 45 days of cryptorchidism. Determinations of the enzyme were made by a biological method of assay—the "spreading reaction." Utilizing a turbidimetric method of assay, Leonard *et al.* (1946, 1948) reported a decrease of the enzyme content in testes of hypophysectomized animals and in cryptorchid animals. Riisfeldt (1949a), employing a viscosimetric assay, described an increase of the enzyme content in testes of adult rats treated with 0.25 to 2.0 mg. testosterone propionate twice weekly for two or four weeks. A decrease of hyaluronidase concentration in testes of rats treated with diethylstilbestrol was reported by Greif (1954), in a study utilizing a colorimetric method of enzyme determination.

Review of the literature reveals that no systematic study using a single method of extraction and enzyme determination of testes under various experimental conditions has been carried out. Furthermore, the lack of an acceptable standard for hyaluronidase activity renders impossible a satisfactory comparison of the results obtained in the various earlier studies.

The purpose of this investigation has been to examine the changes in hyaluronidase concentration in testes of cryptorchidized, hypophysectomized, estrogen-treated, and testosterone-treated rats, employing throughout the study a standard method of extraction and determination of the enzyme. The changes in enzyme concentrations have been correlated with concurrent histological changes in the testes.

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MATERIALS AND METHODS

Two groups of animals were used in this study, one consisting of 127 adult male Long-Evans rats raised in our laboratory and the other of 76 hypophysectomized and 7 intact adult Sprague-Dawley males obtained from the Hormone Assay Laboratories in Chicago.

The enzyme concentrations in normal testes from rats of these two strains did not differ significantly (87.2 ± 5 units/gm. for Long-Evans and 86 ± 1 units/gm. for Sprague-Dawley rats).

Testicular tissue for histological studies was fixed in Bouin's solution, embedded in paraffin and cut at 6 micra. All tissues were stained employing the Masson or Gomori trichrome techniques.

I. *Preparation of Testicular Extract for Hyaluronidase Assay*

The animals were sacrificed by an overdose of ether. Testes were removed rapidly and weighed on a Roller-Smith torsion balance. Samples for histological studies were procured and the remaining tissue was freed from the tunica albuginea and large vessels. Testicular pulp thus obtained was placed in a chilled beaker.

Testicular tissue from 2 to 6 animals was pooled, the total weight determined, and homogenized in a test tube employing a Cenco Stirrer with a glass-rod homogenizer. The homogenized tissue was poured into a beaker, and the test tube and the glass-rod homogenizer were rinsed with 0.1N acetic acid, the rinsing fluid being added to the homogenate. The volume of acetic acid used for rinsing was approximately 1 to 1.5 times the volume of the tissue. This mixture was stirred (using a mechanical stirrer) for four hours, after which time it was centrifuged. The clear supernatant was decanted and stored at 4° C. To the sediment 1 to 1.5 volume of 0.1N acetic acid was added and the extraction procedure repeated two more times. The extracts were combined and their total volume determined. Assays were performed within 48 hours, during which time the extracts were stored at 4° C.

It was found that the first extraction yields approximately 57%, the second 38% and the third 5% of the total enzyme extractable from a given amount of tissue. (A fourth extraction failed to yield further enzymatic activity).

II. *Assay of Hyaluronidase*

A. Stock solutions.

a. Two buffers were used, both made from stock solutions of 0.5M sodium acetate and 0.5M acetic acid:

1. 0.5M acetate buffer, pH 4.2 (130 ml. of 0.5M sodium acetate were combined with 370 ml. of 0.5M acetic acid.)
2. 0.1M sodium acetate buffer, pH 6.0. (97 ml. of 0.5M sodium acetate were combined with 3 ml. of 0.5M acetic acid and 4.384 gm. of sodium chloride, and diluted to a volume of 500 ml. with distilled water; this provides the buffer with 0.15M sodium chloride, as recommended by Tolksdorf (1949)).

b. Albumin. Twenty gm. of "Armour" bovine plasma albumin Fraction V were dissolved in 100 ml. of the 0.5M sodium acetate buffer and the pH of the solution was adjusted to 3.1 with 4N HCl. The solution was then distributed in test tubes (30–50 ml.), immersed in a boiling water bath, kept at this temperature for 30 minutes, and then rapidly cooled by immersing the tubes in ice water. This solution can be kept at 4° C. for 3 to 4 weeks.

c. Substrate. The hyaluronic acid was prepared by the method described by Meyer (1947). The acid was dissolved in 0.1M sodium acetate buffer in a concentration

of 0.4 mg. per ml. Using this concentration we obtained substrate turbidity readings of 160–170, which were found to be optimal for our assay procedure.

d. Hyaluronidase. Schering hyaluronidase[2] was used as the reference standard. The enzyme was dissolved in 0.1M sodium acetate buffer in a concentration of one unit per 0.1 ml. of the buffer. This was stored for not longer than 2 weeks at 4° C.

e. Extract. Immediately before use the extract was diluted with 0.1M sodium acetate buffer. The dilution factor had to be established experimentally for each extract sample. A dilution factor which provided a concentration of 5 to 7 units of hyaluronidase activity in one ml. of the extract was considered adequate, since this concentration was found to be optimal for the assay.

B. Technique of assay. Determinations of hyaluronidase were performed using a modification of the turbidimetric method described by Tolksdorf (1949). The testicular extracts were assayed for their enzymatic activity against the reference standard.

Seven pairs of 15 ml. serological test tubes were used for each assay. Appropriate amounts of hyaluronic acid and 0.1M sodium acetate buffer were placed in each tube, and the tubes immersed in a constant temperature water bath which was kept at

TABLE 1. ARRANGEMENT OF TUBES USED IN HYALURONIDASE ASSAYS

Tube pairs	Substrate	Enzyme	pH 6 buffer
		Standard enzyme	
1	0.5 cc.	0.0 cc.	0.5 cc.
2	0.5 cc.	0.1 cc.	0.4 cc.
3	0.5 cc.	0.2 cc.	0.3 cc.
4	0.5 cc.	0.3 cc.	0.2 cc.
		Extract solution	
5	0.5 cc.	0.1 cc.	0.4 cc.
6	0.5 cc.	0.2 cc.	0.3 cc.
7	0.5 cc.	0.3 cc.	0.2 cc.

37° C. when temperature equilibrium was reached, standard enzyme and extract were added to the respective test tubes, the solutions mixed and incubated in the water bath for 30 minutes. Solutions were placed in the test tubes in the amounts and combinations indicated in Table 1.

The enzymatic reaction was terminated by addition of 3 ml. of cold 0.5M acetate buffer to each tube (Meyer, 1952). This was followed by addition of 1 ml. of acidified albumin solution. The contents of the tubes were mixed and turbidity was allowed to develop at room temperature for 30 minutes. The developed turbidities were read in a Klett-Summerson colorimeter using a blue filter. The colorimeter was adjusted to zero with a blank containing 1 ml. of albumin solution and 4 ml. of 0.5 M acetate buffer.

C. Calculations. Turbidity readings of samples containing the standard enzyme were plotted and a standard curve obtained. The enzyme concentrations of the extract samples were determined by interpolation on the standard curve. The values so obtained were averaged and the total amount of enzyme in the combined extracts determined. This value was divided by the number of grams of testicular tissue employed in the extraction. The resulting value was the amount of enzyme per gram of wet testicular tissue expressed in units of Schering hyaluronidase.

[2] "Hyronase," a brand of hyaluronidase, was generously supplied by Dr. Edward Henderson, Schering Corporation, Bloomfield, N.J.

EXPERIMENTAL

Hypophysectomized series (Table 2, Figure 1).

It is evident from the curve in Figure 1 that the decrease in hyaluronidase concentration follows closely the diminution in testicular weight. A significant drop in enzyme concentration was observed on the 10th day following hypophysectomy, the decrease following a precipitous course until the 20th day when the enzyme concentration was 20% of normal. During the following 10 days the rate of diminution was less rapid, the enzyme concentration on the 30th day after hypophysectomy being approximately 9% of normal.

Histological studies revealed essentially normal spermatogenesis in animals sacrificed 6 days following hypophysectomy, but definite changes were present in testes obtained 10 days after hypophysectomy (Fig. 4). Although all stages of spermatogenesis were present, the tubules were decreased in size, the number of maturing sperm appeared to be decreased, many spermatids were desquamated or pycnotic and spermatocytes showed an apparent increase in numbers. Related to this the number of spermatocytic divisions was decreased. This picture is one of arrest or slowing of maturation of the spermatocytes. The Leydig cells were markedly reduced in numbers.

Testes of animals sacrificed 15 days following hypophysectomy showed very marked alteration of the spermatogenic process. In most tubules stages beyond secondary spermatocytes were absent and some tubules contained cells up to the primary spermatocyte stage only. The lumens of the tubules were filled in most instances with debris and sloughed cells. Some tubules contained occasional scattered sperm. The tubular diameter was markedly decreased, and Leydig cells were very atrophic. In rats sacrificed 20 days following hypophysectomy the tubules contained chiefly cells up to the stage of primary spermatocytes, although some tubules

TABLE 2. CHANGES IN HYALURONIDASE CONCENTRATION IN RAT TESTES FOLLOWING HYPOPHYSECTOMY

Days after hypophysectomy	No. of rats	No. of assays	Body wt., gm.	Testicular wt., mg.	Hyaluronidase content units per gm.
6	8	4	210 ± 11.5*	2590 ± 91	79.0 ± 5.9
10	12	6	203 ± 3.8	2272 ± 72	63.0 ± 6.25
12	8	4	207 ± 3.6	2033 ± 57	39.0 ± 9.2
15	13	6	213 ± 4.5	1432 ± 54	26.6 ± 4.7
20	10	4	199 ± 2.2	941 ± 44	17.0 ± 4.0
25	4	1	199 ± 5.6	655 ± 50	14.0 ± 0.0
30	8	3	201 ± 4.7	497 ± 34	9.7 ± 1.4
Normal controls Sprague-Dawley	11	6	220 ± 5.8	2975 ± 155	86.0 ± 1.0

* Mean ± standard error of the mean.

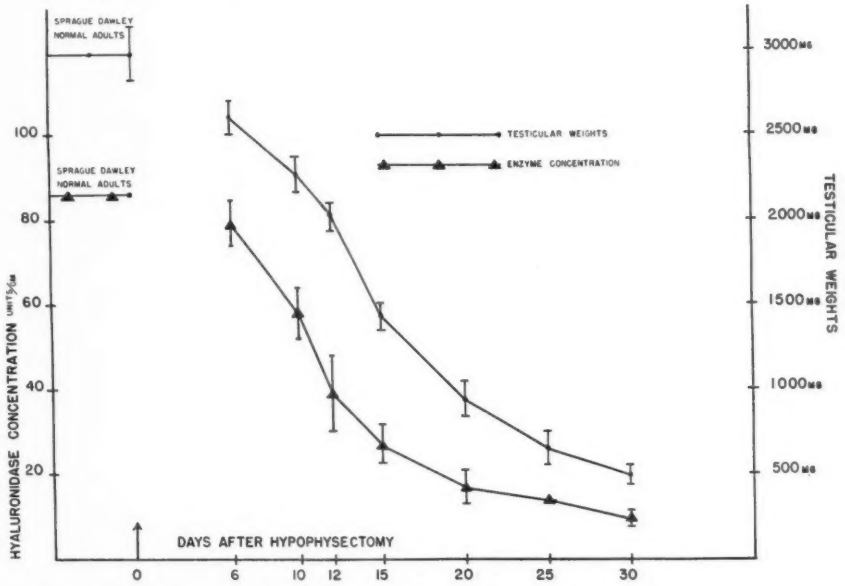

FIG. 1. Enzyme concentration and testicular weights of hypophysectomized rats.

contained secondary spermatocytes. Mitotic activity was observed in some primary spermatocytes, whereas others appeared to be undergoing pycnosis or fragmentation. The lumens of the tubules were commonly filled with debris and in some testes a few, apparently old, sperm were seen. Testes of animals sacrificed 25 or 30 days (Fig. 5) after hypophysectomy revealed similar pictures except that very few tubules contained cells (secondary spermatocytes) beyond the primary spermatocyte stage, and these cells frequently appeared to be abnormal. The process of spermatogenesis to all intents and purposes did not proceed beyond the primary spermatocyte stage. Leydig cells were absent in these testes, as is true of others beyond 15 days after hypophysectomy.

Cryptorchid series (Table 3, Figure 2)

The drop in hyaluronidase concentration in testes from these animals did not follow closely the decrease in testicular weight. It is evident from the data that a significant drop in hyaluronidase concentration occurred as early as the 4th postoperative day although testicular weights remained within normal limits. Between the 4th and 5th postoperative day no further significant change in hyaluronidase concentration occurred, but a marked drop in testicular weight became evident. A precipitous drop in enzyme concentration was observed between the 6th and 10th day; on

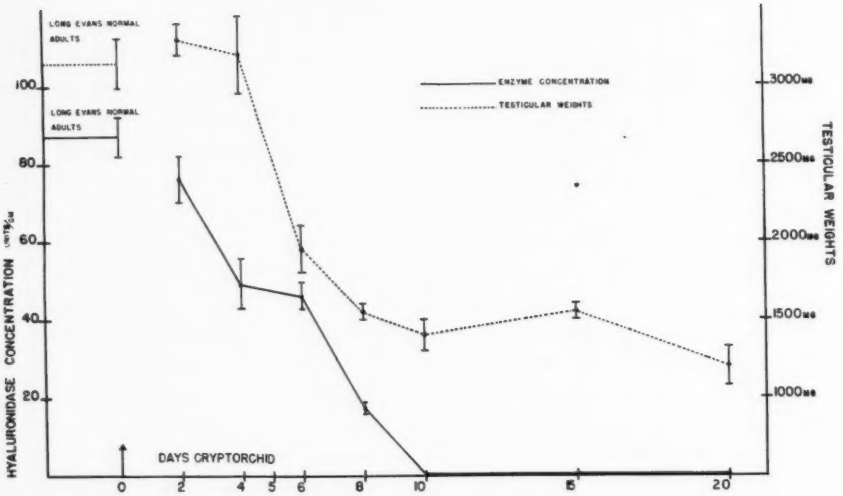

FIG. 2. Enzyme concentration and testicular weights of cryptorchid rats.

the latter day, as well as on the 15th day, no enzyme (or only traces of it) being present. On the 20th postoperative day enzyme was completely undetectible. Testicular weight decreased significantly between the 6th and 10th postoperative days, leveling off after the 10th day with little further change evident on the 20th day.

Changes in the histological picture of the testes of cryptorchidized rats could be detected already on the second postoperative day. Although most tubules contained mature sperm and all other stages of spermatogenesis, some tubules showed marked sloughing of cells of all stages except spermatogonia. Mitotic activity in the primary spermatocytes appeared to be depressed, with virtually no normal mitoses being seen. Testes of animals sacrificed on the 4th postoperative day (Fig. 6) revealed progressive damage of the type described, some tubules containing no cells beyond pri-

TABLE 3. EFFECT OF CRYPTORCHIDISM ON HYALURONIDASE CONCENTRATION IN RAT TESTES

Days after operation	No. of rats	No. of assays	Body wt., gm.	Testicular wt., mg.	Hyaluronidase content, units per gm.
2	5	3	342 ± 20*	3350 ± 81.0	76.0 ± 5.6
4	6	5	359 ± 17	3207 ± 242.0	49.0 ± 6.3
6	12	5	335 ± 31	1925 ± 118.0	46.0 ± 2.9
8	3	3	283 ± 7	1541 ± 17.4	17.3 ± 1.2
10	8	3	302 ± 10	1413 ± 80.0	Traces → 0
15	4	2	413 ± 35	1542 ± 56	Traces → 0
20	4	1	227 ± 11	1281 ± 129	0
Normal controls (Long-Evans)	35	18	391 ± 37	3150 ± 165	87.2 ± 5.0

* Mean ± Standard error of the mean.

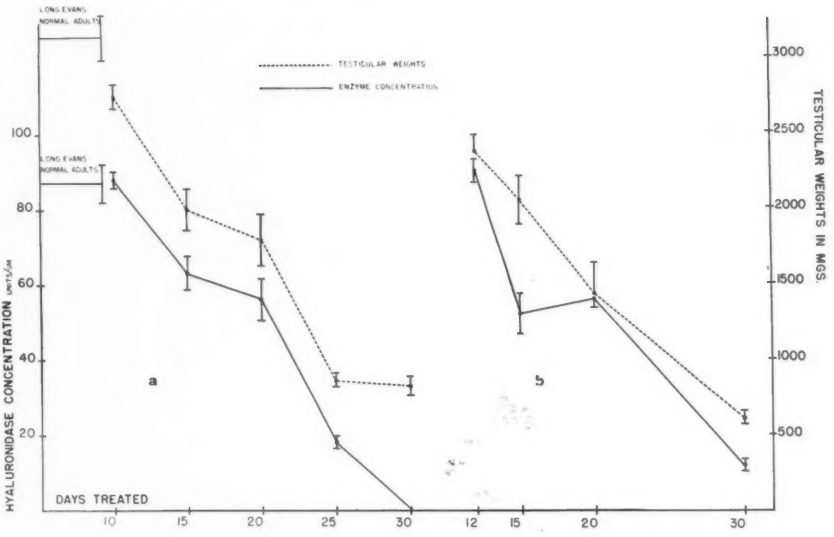

FIG. 3. a) Enzyme concentration and testicular weights of estradiol treated rats. b) Enzyme concentration and testicular weights of diethylstilbestrol treated rats.

mary spermatocytes, while others appeared to be essentially unchanged with maintenance of orderly spermatogenesis. In tubules where primary spermatocytes showed mitotic activity this process appeared abnormal. The most seriously damaged tubules showed a peculiar vacuolation centered chiefly in areas occupied by primary spermatocytes. Leydig cells appeared normal.

On the 6th postoperative day there was marked sloughing of the germinal epithelium in the majority of tubules, including frequently even the primary spermatocytes. The lumens were filled with cellular debris, sloughed germinal cells, giant cells, and sperm in various stages of maturation. The primary spermatocyte layer contained large vacuoles and normal mitoses could not be detected. Occasional tubules appeared relatively unaffected, but these were exceptional. Leydig cells appeared to be increased in numbers and marked intertubular edema was evident. On the 8th postoperative day the tubules appeared to be affected more uniformly, most of them containing Sertoli cells, spermatogonia and primary spermatocytes, but no advanced stages. The lumens of some tubules contained masses of sloughed germinal elements, including mature sperm, and giant cells. In animals sacrificed ten days after the operation (Fig. 7) the seminiferous tubules contained only Sertoli cells, spermatogonia and occasional spermatocytes, except for some tubules which had lumens filled with debris and degenerated germinal cells. Mature sperm free in the lumens or at-

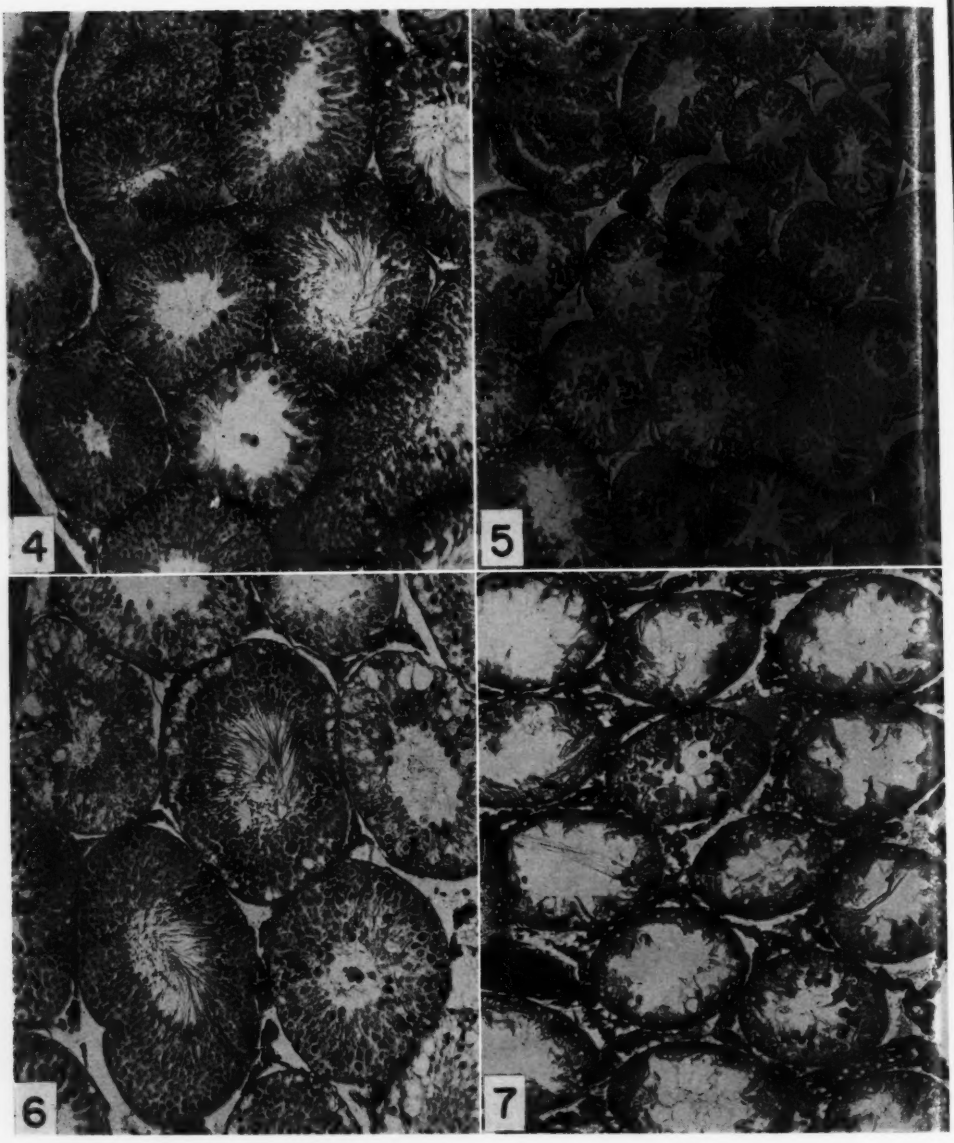

PLATE I

FIG. 4. Testis from a rat sacrificed 10 days after hypophysectomy. ×115.
FIG. 5. Testis from a rat sacrificed 30 days after hypophysectomy. ×115.
FIG. 6. Testis from cryptorchid rat sacrificed 4 days after the operation. ×115.
FIG. 7. Testis from cryptorchid rat sacrificed 10 days after the operation. ×115.

tached to Sertoli cells were seen occasionally. Intertubular edema was evident. On the 15th postoperative day the histological condition was quite similar to that seen after 10 days. It is noteworthy that a number of mature sperm attached to the Sertoli cells could still be detected. On the 20th day most of the tubules contained Sertoli cells, a few spermatogonia and occasional primary spermatocytes. The lumens were empty, the sloughed elements having been lost by passage out of the testes. Intertubular edema was usually pronounced. Leydig cells showed the abundance commonly noted in the cryptorchid condition.

Estrogen series (Table 4)

In these experiments animals were divided into two major groups. One was treated with daily subcutaneous injections of 200 μg. α-estrodiol in oil (Fig. 3a), the other group with daily injections of 1 mg. diethylstilbestrol in oil (Fig. 3b).

The enzyme concentration curve of α-estradiol-treated rats followed closely that of corresponding testicular weights, a significant drop in enzyme concentration occurring on the 15th day of treatment. The most rapid decrease was observed between the 20th and 25th day and on the 30th day only traces of the enzyme were present (Fig. 3a).

The curves for enzyme concentration and corresponding testicular weights in diethylstilbestrol treated animals were comparable to those seen in animals injected with α-estradiol (Fig. 3b), the small differences being probably of no significance.

Histological pictures were essentially the same in both groups, the

TABLE 4. EFFECT OF ESTROGEN TREATMENT ON HYALURONIDASE CONCENTRATION IN RAT TESTES

Days treated	No. of rats	No. of assays	Body wt., gm.	Testicular wt., mg.	Hyaluronidase content, units per gm.
α-Estradiol, 200 μg. daily					
10	5	3	265 ± 5*	2730 ± 76	88.0 ± 1.9
15	12	6	264 ± 12.4	1983 ± 121	63.0 ± 4.3
20	7	5	286 ± 5.6	1793 ± 160	55.2 ± 5.5
25	12	4	304 ± 7	860 ± 28	18.2 ± 1.7
30	5	1	282 ± 12	831 ± 55	Traces
Diethylstilbestrol 1.0 mg. daily					
12	4	3	267 ± 7.5	2365 ± 104	90.0 ± 2.9
15	4	3	306 ± 18.7	2053 ± 154	52.0 ± 5.2
20	4	3	295 ± 15.4	1430 ± 212	56.0 ± 2.2
30	4	4	278 ± 4.8	618 ± 34	12.0 ± 1.2
Normal controls (Long-Evans)					
—	35	18	391 ± 37	3150 ± 165	87.2 ± 5.0

* Mean ± Standard error of the Mean.

TABLE 5. EFFECTS OF TESTOSTERONE PROPIONATE AND α-ESTRADIOL ON TESTICULAR HYALURONIDASE CONCENTRATION IN NORMAL AND HYPOPHYSECTOMIZED RATS

Treatment	No. of rats	No. of assays	Body wt., gm.	Seminal vesicles wt., mg.		Prostatic wt., mg.	Testicular wt., mg.	Hyaluronidase content, units per gm.
				Full	Empty			
Normal rats (Long-Evans strain)								
2 mg. T.P. twice a week for 4 weeks	9	4	350 ± 18*	936 ± 50	336 ± 15	666 ± 21	2750 ± 58	79.0 ± 4.8
10 mg. T.P. daily for 3 weeks	4	3	302 ± 13	1505 ± 82	512 ± 30	635 ± 47	2823 ± 43	89.3 ± 5.5
10 mg. T.P. daily for 4 weeks	8	4	327 ± 22	1575 ± 61	530 ± 22	819 ± 31	2810 ± 49	91.0 ± 3.0
Non-treated controls	35	18	391 ± 37	782 ± 81	321 ± 18	510 ± 29	3150 ± 165	87.2 ± 5.0
Hypophysectomized rats (Sprague-Dawley strain)								
2 mg. T.P. daily for 12 days	4	3	200 ± 5	745 ± 28	312 ± 34	298 ± 21	2735 ± 33	91.0 ± 1.0
2 mg. T.P. daily for 20 days	3	3	265 ± 8	1190 ± 68	440 ± 28	601 ± 40	2770 ± 41	82.0 ± 6.1
2 mg. T.P. daily for 40 days	4	3	198 ± 4	1362 ± 53	457 ± 35	987 ± 34	2247 ± 28	88.0 ± 4.2
2 mg. T.P. daily for 20 days, and 2 mg. T.P. and 100 μg. α-estradiol daily for another 20 days	4	3	200 ± 7	1696 ± 48	526 ± 25	1136 ± 48	2430 ± 30	91.0 ± 2.8

* Mean ± Standard error.

earliest changes of significance occurring on the 15th day of treatment. At this time Leydig cells were atrophic, mitotic activity of the spermatocytes was decreased, sperm maturation was markedly reduced and numbers of pycnotic spermatocytes and spermatids were evident. On the 20th day of treatment the germinal epithelium showed further alteration marked by widespread arrest of spermatogenesis at the primary spermatocyte stage with occasional tubules only showing secondary spermatocytes. Testes of animals sacrificed on the 30th day of treatment revealed generalized spermatogenic arrest at the stage of primary spermatocytes so that most of the tubules contained no elements beyond primary spermatocytes. Occasional tubules showed a few secondary spermatocytes, spermatids and sperm being completely absent. Many tubules presented a decreased number of normal primary spermatocytes, together with numerous pycnotic, multinucleated and otherwise abnormal cells. The tubules were markedly decreased in size and functional Leydig cells were absent.

Testosterone series (Table 5, Figs. 8–11).

Testosterone propionate (T.P.)[3] was administered subcutaneously to normal adult males in doses from 2 mg. twice weekly to 10 mg. per day for periods of time ranging from 10 to 45 days. The series in which the dose was 2 mg. twice weekly was examined to duplicate Riisfeldts studies. Hypophysectomized rats received 2 mg. T.P. per day for 12, 20 and 40 days respectively. One group of hypophysectomized animals was treated

[3] We should like to acknowledge the kindness of Dr. Robert Gaunt of Ciba Pharmaceutic Products, Summit, N.J., for furnishing the testosterone propionate and α-estradiol used in these studies.

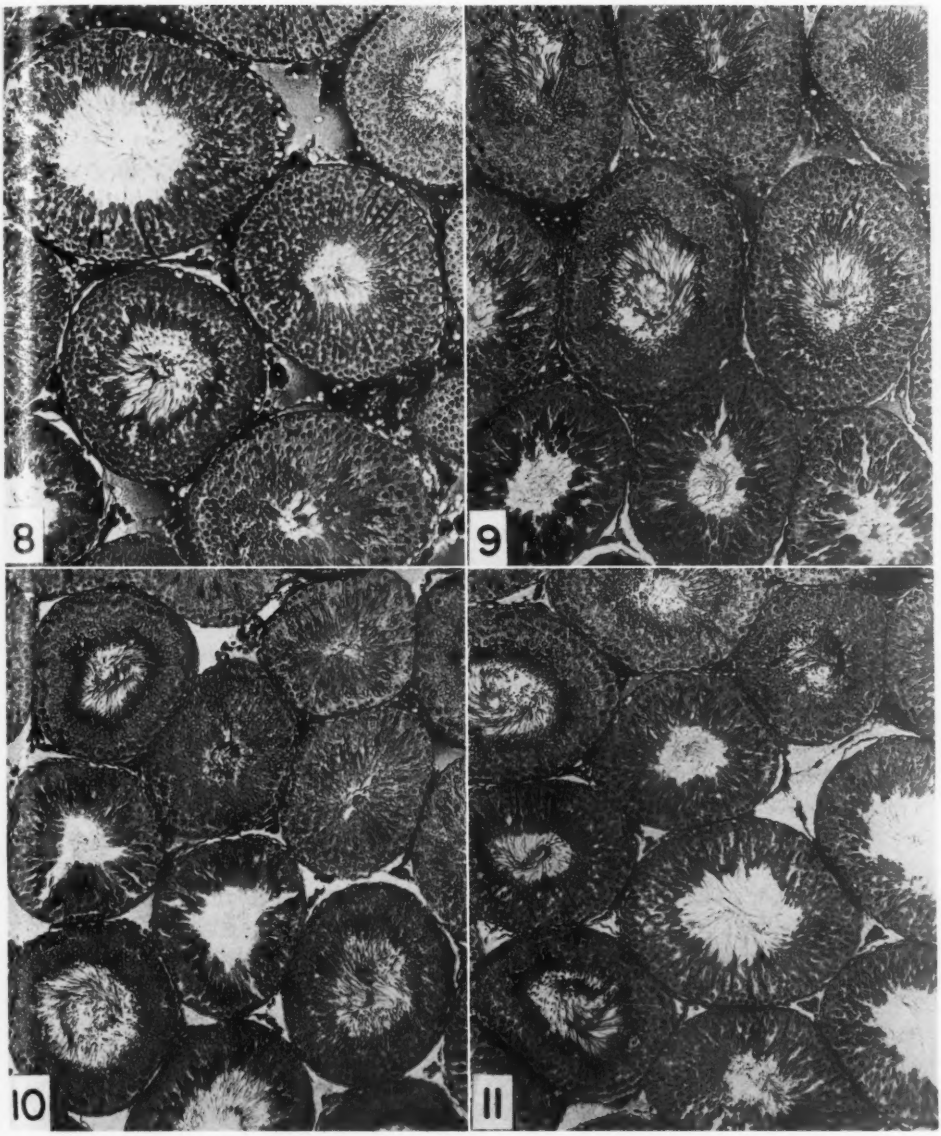

PLATE II

FIG. 8. Normal adult rat testis. ×115.

FIG. 9. Testis of normal adult rat treated with 10 mg. T.P. daily for 4 weeks. ×115.

FIG. 10. Testis of hypophysectomized rat treated with 2 mg. T.P. daily and sacrificed 40 days after hypophysectomy. ×115.

FIG. 11. Testis of hypophysectomized rat treated with 2 mg. T.P. daily for 20 days, and 2 mg. T.P. and 100 estradiol daily for another 20 days. ×115.

with 2 mg. T.P. per day for 20 days, and for an additional 20 days with T.P. and 100 μg. α-estradiol per day.

The enzyme concentration in testes of normal rats treated with T.P. remained in all cases within normal limits (Table 5) and the same was true when hypophysectomized rats were given T.P. The addition of estradiol did not alter the effect of T.P. on enzyme concentration (Table 5) in hypophysectomized rats. The effectiveness of the doses of T.P. can be judged further by the increased weight of the accessory organs (Table 5).

Histological studies of the testes in T.P. treated intact rats revealed no significant changes in tubular morphology and spermatogenesis (Figs. 8 and 9). The Leydig cells were decreased in numbers or completely absent, depending on the dose of T.P. and length of treatment. The testes of hypophysectomized animals (Fig. 10) were adequately maintained on the dose of T.P. employed and the addition of estrogen to T.P. had no effect on the histological picture (Fig. 11).

DISCUSSION

That hyaluronidase concentration in rat testes is related to the functional status of the germinal epithelium has been shown by several investigators, but any attempt to integrate these studies has been seriously hampered because of the widely different methods employed in enzyme estimation. Thus Sprunt *et al.* (1938) utilized a biological method, Leonard *et al.* (1946, 1948) a turbidimetric, Riisfeldt (1949a and b) a viscosimetric and Greif (1954) a colorimetric method of assaying enzyme levels. At the present time there is no way of comparing satisfactorily the results obtained by the use of these various methods of hyaluronidase assay. Furthermore, the fact that markedly different methods of enzyme extraction were utilized by these investigators provides additional complications since the amount of enzyme recovered varies greatly with each method. Indeed, some of the procedures appear to have been quite inefficient in obtaining adequate samples of the enzyme.

In the present studies we have taken care to employ uniform assay and extraction procedures throughout. We believe that this provides a more justifiable basis for comparing the effects of various experimental conditions on the level of testicular hyaluronidase. The extraction procedure is simple, eliminates to a high degree the loss of enzyme that may have occurred with other extraction procedures and provides for satisfactorily reproducible results.

In testes from hypophysectomized rats Leonard *et al.* (1948) reported a significant drop of hyaluronidase concentration by the 24th day after hypophysectomy. This observation would seem to suggest that a drop in enzyme concentration does not occur until hypophysectomy imposed atrophy of the germinal epithelium is far advanced since it is possible to recognize definite microscopic changes by the 10th day after hypophy-

sectomy. In our studies it was possible to demonstrate a significant drop in enzyme concentration as early as the 10th day after pituitary removal, an observation which correlates well with the histologic picture of early but definite damage to the germinal epithelium. At this time all stages of the spermatogenic process are present, but the lack of gonadotrophic factors is manifested by reduction in the number of maturing sperm and of normal cells beyond the primary spermatocyte stage. This would suggest that the more advanced stages are more closely related to hyaluronidase formation than are spermatogonia and primary spermatocytes. The gradual decrease of the enzyme content subsequent to the 10th day paralleled the diminution in testicular weight as well as the progressive loss of all cells beyond primary spermatocytes. This may be regarded as substantiating evidence for relating hyaluronidase production to the advanced stages of spermatogenesis, but can hardly be regarded as conclusive proof since the presence of intact and apparently normal spermatogonia and primary spermatocytes does not necessarily indicate that the metabolic processes of these cells, including the possible production of enzymes, were unimpaired.

Treatment with estrogen produces effects both on enzyme content and testicular histology similar to those seen in hypophysectomized animals, except that the changes occurred a few days later. This is not surprising since the result of such treatment is in effect a physiological hypophysectomy in so far as the production of gonadotrophic homones is concerned. It is to be expected that suppression of gonadotrophin secretion by estrogen would require a few days as compared to its abrupt termination in hypophysectomized animals. There is no reason to believe that estrogen *per se* has a direct effect on the production of hyaluronidase. Indeed, present observations in animals treated with both estrogen and androgen offer evidence to the contrary.

Cryptorchidism produces testicular damage much more rapidly than does hypophysectomy or treatment with estrogens. Thus we were able to detect a significant drop in enzyme concentration on the 4th postoperative day, this corresponding to definite histologic changes in the germinal epithelium. These changes affect some tubules more rapidly than others during the first week after operation, but even on the 4th day many tubules show considerable sloughing of cells down to spermatocytes and a lack of normal meiotic activity in the latter cells. These defects soon appear in all tubules so that following the 15th day, by when hyaluronidase had disappeared, the cellular population of the tubules is confined to spermatogonia, Sertoli cells and reduced numbers of primary spermatocytes. Leydig cells appear to be quite normal in all respects.

Riisfeldt (1949a) employing a viscosimetric method of hyaluronidase determination, reported an increase in the amount of testicular hyaluronidase and stimulation of the spermatogenic process in intact adult rats fol-

lowing testosterone propionate treatment. We have not been able to reproduce Riisfeldt's results in any respect. After treatment with T.P. not only did the concentration of enzyme remain unchanged, but the spermatogenic process showed no evidence of stimulation. The testes of hypophysectomized rats treated with T.P., were maintained at essentially normal weights, exhibited normal spermatogenesis and contained concentrations of enzyme entirely comparable to those found in normal rats. We have no explanation for the discrepancy between our results and those of Riisfeldt, particularly in view of some recent preliminary studies in which we have examined the enzyme content of testes from T.P. treated rats as measured by the viscosimetric method described and used by Riisfeldt. No change in enzyme levels was detected in that study.

On the whole the evidence provided by these studies would suggest that the Sertoli cells, the Leydig cells, spermatogonia and primary spermatocytes are less likely to be of primary importance to the production of hyaluronidase than are the secondary spermatocytes, spermatids and sperm, since reduction in the numbers of these latter cells in various experimental states coincides with the drop in level of the enzyme.

Sertoli cells retain at least morphologic normality in each of the several experimental conditions and seem to have little if any direct relationship to hyaluronidase production. The same seems true for the Leydig cells since their condition bore no relationship to the levels of enzyme. In animals of the cryptorchid series they were morphologically and functionally normal, whereas in hypophysectomized and estrogen-treated animals they were atrophic. In each of these groups hyaluronidase levels were low. Of even greater significance was the absence of Leydig cells in normal and hypophysectomized animals treated with T.P., since in each of these groups enzyme concentration was normal.

Spermatoginia, while not immune to the various experimental procedures used in this study, appear to be unlikely sites of hyaluronidase production, since their presence is constant in all of these conditions. Furthermore, it has been observed (Riisfeldt, 1949a) that hyaluronidase is absent from the testes of rats until spermatogenesis proceeds beyond the spermatogonium stage.

It is more difficult to dismiss the primary spermatocyte as an important factor in the production of hyaluronidase. Although these cells are present in all of the conditions we have studied here, regardless of the level of enzyme, it is not possible to ignore the fact that the important meiotic process is interfered with by cryptorchidism, hypophysectomy and estrogen treatment and that other functions of the sermatocyte also may suffer. Other experiments and observations must be made to provide critical information on this point.

The remaining three cell types, secondary spermatocytes, spermatids and spermatozoa, appear to have a closer relationship to hyaluronidase

production since it is these cells that become less prominent as the level of hyaluronidase decreases. Of the three the secondary spermatocytes probably can be dismissed more readily than the other two. Their life is short, a very brief interval between an origin from primary spermatocytes and a division to form spermatids, and it is not likely that they serve an important function in the production of the enzyme. It is worth remembering, too, that in hypophysectomized and estrogen-treated animals secondary spermatocytes, although much reduced in numbers, are not completely absent although the enzyme content is reduced essentially to zero.

On the basis of the evidence at hand the spermatid seems to be the testicular cell that has the most significant role in the production of hyaluronidase. The most marked decreases in enzyme levels in our animals occurred at times when spermatid formation and metamorphosis suffered significant impairment. Furthermore, the spermatid has a relatively long period of existence during its metamorphosis into a spermatozöon.

Spermatozoa are simply the product of spermatid metamorphosis and under normal circumstances are detached rapidly from the Sertoli syncytium to be carried to the epididymis. It would appear unlikely that the mature spermatozöon is intimately related to the actual formation of testicular hyaluronidase.

Although it would seem that the spermatid probably plays the most significant role in the production of the enzyme, it will be necessary to assess other testicular conditions to provide essential and critical information before a more than tentative conclusion can be made.

SUMMARY

A method of extraction and assay of testicular hyaluronidase has been described and used in the determination of enzyme levels in testes of rats under several experimental conditions.

Utilizing these procedures, testes of normal adult rats were found to contain 87.2 ± 5.0 units per gram.

After hypophysectomy the enzyme content showed an initial decrease on the 10th day and reached a low value of 9.7 ± 1.4 units per gram on the 30th day.

The enzyme content of experimentally cryptorchid testes diminished significantly by the 4th day after the operation and was substantially zero by the 10th day.

The hyaluronidase levels of testes from rats treated with estradiol or diethylstilbestrol showed an initial decrease on the 15th day and reached or approached zero by the 30th day.

Testosterone propionate did not alter the levels of enzyme in normal animals, but did maintain normal levels in hypophysectomized and hypophysectomized-estrogen-treated rats.

The histological condition of testes in the various experimental states has been described and an attempt has been made to relate hyaluronidase production to specific cellular constituents of the testes.

REFERENCES

CHAIN, E. AND E. S. DUTHIE: *Nature* **144:** 977. 1939.
CHAIN, E. AND E. S. DUTHIE: *Brit. J. Exper. Path.* **21:** 324. 1940
DURAN-REYNALS, F.: *Compt. rend. soc. biol.* **99:** 6. 1928.
DURAN-REYNALS, F.: *J Exper. Med.* **50:** 327. 1929.
GREIF, R. L.: *J. Biol. Chem.* **206:** 381. 1954.
LEONARD, S. L., P. L. PERLMAN AND R. KURZROK: *Endocrinology* **39:** 261. 1946.
LEONARD, S. L., P. L. PERLMAN AND R. KURZROK: *Endocrinology* **42:** 176. 1948.
MEYER, K., R. DUBOS AND E. SMITH: *J. Biol. Chem.* **118:** 71. 1937.
MEYER, K.: *Physiol. Rev.* **27:** 335. 1947.
MEYER, K.: Personal communication. 1952.
RIISFELDT, O.: *Endocrinology* **45:** 622. 1949a.
RIISFELDT, O.: *Acta endocrinol.* **3:** 137. 1949b.
SPRUNT, D. H., C. W. HOOKER AND J. S. RAPER: *Proc. Soc. Exper. Biol. & Med.* **41:** 398. 1939.
TOLKSDORF, S., M. MCCREADY, D. R. MCCULLAGH AND E. SCHWENK: *J. Lab. Clin. Med.* **34:** 74. 1949.

EFFECT OF PROPYLTHIOURACIL ON THE RATIO OF THE RADIOIODIDE CONCENTRATIONS IN THYROID GLAND AND SERUM IN NORMAL AND HYPOPHYSECTOMIZED RATS

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RECENT studies by Wollman and Scow (1953) on mice indicated that when binding of iodine was blocked by a single injection of propylthiouracil the ratio of the radioiodide concentrations in the thyroid gland and serum (T/S ratio) was greater than when binding was permitted. This paper reports similar data for normal and hypophysectomized rats.

METHODS

Experiments were performed on male Holtzman rats, 3 months of age. The rats were fed a lactating diet[2] (Scow, 1953) since weaning. The iodine content of this diet was not measured. It was not low enough to cause thyroid enlargement (Table 1), but was low enough to yield rats with active thyroids as evidenced by high radioiodine uptakes and high T/S ratios. Rats were 30 days of age when they were hypophysectomized through the parapharyngeal approach. Completeness of hypophysectomy was ascertained by visual inspection of the region of the sella turcica for pituitary fragments using a binocular microscope at 9× magnification.

The T/S ratio was measured by methods similar to those described earlier in the studies on mice with the exception of a few details. One hour before radioiodide injection normal rats were given 10 mg. of propylthiouracil and hypophysectomized rats were given 5 mg. to block binding. Twenty μc. of radioiodide[3] were injected intraperitoneally. Samples of thyroid and blood were obtained from normal rats at one hour after radioiodide injection and from hypophysectomized rats at two hours. Homogenates of thyroid glands were deproteinized using 0.4 ml. each of the $Ba(OH)_2$ and $ZnSO_4$ reagents. Radioiodine content of all samples was measured by gamma counting using well-type Geiger counters.

RESULTS

The thyroid uptake of I^{131} in animals not treated with propylthiouracil was approximately 4.7 % of the dose in normals at one hour after radio-

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[1] National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[2] Composition of diet: Ground whole wheat, 67.5%; casein (crude), 15%; whole milk powder, 10%; cottonseed (Wesson) oil. 5.25%; $CaCO_3$, 1.5%; NaCl (reagent), 0.75%; Natola (Parke, Davis and Co.), 212.5 mg./kg. diet (supplying 11,770 USP units Vitamin A and 2,340 USP units Vitamin D/kg. diet); and 50 mg. Vitamin E/kg. diet.

[3] The carrier-free I^{131} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

iodide injection and 0.22% in hypophysectomized rats at two hours. The I^{131} content of the thyroids of animals given propylthiouracil was approximately 25% of that of their control animals.

In rats in which binding of iodine was permitted the radioiodide was only a small fraction of the total I^{131} in the thyroid (Table 1). In both normal and hypophysectomized rats the T/S ratio was much larger when organic binding of iodine was blocked by propylthiouracil than when binding was permitted (Table 1).

DISCUSSION

The present values for the ratio of radioiodide content to protein-bound I^{131} content of the thyroid are in fairly good agreement with earlier data by Albert and Lorenz (1951). The small differences are probably largely due to the use of different deproteinizing agents and to the different time intervals investigated.

The T/S ratio in normal rats when binding was blocked by a single dose of propylthiouracil (145) is considerably higher than the value (25) usually reported by others (VanderLaan and VanderLaan, 1947; Halmi,

TABLE 1. THE DEPENDENCE OF THE T/S RATIO ON A SINGLE INJECTION OF PROPYLTHIOURACIL IN NORMAL AND HYPOPHYSECTOMIZED RATS

Group	Number of rats	Dose of propylthiouracil, mg.	Rat weight, gm.	Thyroid weight, mg.	T/S	Radioiodide / Organic I^{131}
Normal	6	10	241 ± 41[1]	14.8 ± 2.1[1]	145 ± 11[2]	—
	6	0	274 ± 24	16.0 ± 1.4	12.7 ± 0.7	0.026 ± 0.001[2]
Hypophysectomized	5	5	94 ± 4	5.9 ± 0.9	8.6 ± 1.5	—
	5	0	101 ± 9	6.1 ± 1.3	1.61 ± 0.09	0.292 ± 0.008

[1] Standard deviation.
[2] Standard error.

1954). This discrepancy may be due to differences in the iodine content of the diets used. The T/S ratio in hypophysectomized rats when binding was blocked by a single dose of propylthiouracil appears to be in good agreement with earlier measurements by Greer (1949) and Halmi (1954).

A large increase in T/S ratio occurred when binding was blocked with propylthiouracil. This effect is similar to that in the mouse (Wollman and Scow, 1953) and suggests, as in the mouse, that the binding of thyroid iodide is a relatively fast process and that equilibration of radioiodide between blood and thyroid gland is rate-limiting for accumulation of radioiodine by the thyroid gland (Wollman and Scow, 1953; Wollman, 1954).

When binding was permitted the T/S ratio (using radioiodide) in normal rats (12.7) was only 1/40 of the corresponding ratio for stable iodide (500) previously reported by Taurog, Tong, and Chaikoff (1951). The difference in these values does not appear to be due to differences in the

physiological state of the thyroid glands in the animals being compared. In particular, the thyroid glands of the rats studied by Taurog, Tong, and Chaikoff (1951) with maximum I^{131} uptakes[4] of 10 to 15% would appear to be less active than those of the present rats. The large difference between the ratio of the stable iodide concentrations in thyroid gland and serum and the corresponding ratio for radioiodide might imply that in the thyroid gland there is a considerable amount of iodide which is not freely exchangeable with blood iodide. The value of the T/S ratio using radioiodide is probably a reliable measure of the freely exchangeable iodide. Its low value cannot be attributed to incomplete equilibration since the freely exchangeable iodide compartment has undoubtedly equilibrated with the blood iodide as evidence by the large ratio of protein-bound I^{131} to radioiodide in the thyroid at one hour after injection of radioiodide (Table 1). On the other hand, the measurement of the ratio using stable iodide may be too high because of the inherent analytical difficulty associated with the determination of small amounts of iodide in the presence of very large amounts of iodine-containing compounds which are slightly unstable. This same analytical difficulty is inherent in direct attempts to demonstrate with radioiodide a slowly-exchangeable iodide compartment in the thyroid gland. It would appear unsafe to conclude at present that a slowly exchangeable iodide compartment exists.

SUMMARY

In both normal and hypophysectomized rats the ratio of the radioiodide concentrations in thyroid gland and serum (T/S ratio) was much higher when organic binding of iodine was blocked by a single dose of propylthiouracil than when binding was permitted. This was considered evidence that the rate of equilibration of radioiodide between thyroid gland and blood was rate-limiting for the over-all accumulation of I^{131} by the thyroid. When binding was permitted the T/S ratio (using radioiodide) was only 1/40 of that reported for the corresponding ratio for stable iodide.

Acknowledgment

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REFERENCES

ALBERT, A. AND N. LORENZ: *Proc. Soc. Exper. Biol. and Med.* **77:** 204. 1951.
GREER, M. A.: *Endocrinology* **45:** 178. 1949.
HALMI, N. S.: *Endocrinology* **54:** 97. 1954.
SCOW, R. O.: *Am. J. Physiol.* **173:** 199. 1953.
TAUROG, A., W. TONG AND I. L. CHAIKOFF: *J. Biol. Chem.* **191:** 677. 1951.
VANDERLAAN, J. E. AND W. P. VANDERLAAN: *Endocrinology* **40:** 403. 1947.
WOLLMAN, S. H.: *Endocrinology* **54:** 35. 1954.
WOLLMAN, S. H. AND R. O. SCOW: *Endocrinology* **53:** 332. 1953.

[4] Personal communication.

EFFECT OF VARIOUS GOITROGENS AND OF DOSE OF PROPYLTHIOURACIL ON THE RATIO OF RADIOIODIDE CONCENTRATIONS IN THE THYROID GLAND AND SERUM IN MICE

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IN SOME recent studies the ratio of the thyroid radioiodide concentration to the serum radioiodide concentration (T/S ratio) was higher when binding of radioiodide was blocked by a single injection of either propylthiouracil (Wollman and Scow, 1953) or iodide (Wollman and Scow, 1954b) than when binding was permitted. These observations are in agreement with conclusions reached in a theoretical analysis (Wollman, 1954) that merely blocking binding of radioiodide would be expected to elevate the T/S ratio provided that the iodide binding rate was fast compared with the rate of equilibration of iodide between thyroid gland and blood. It was also recognized (Wollman, 1954) that a goitrogen might elevate the T/S ratio by some mechanisms in addition to that of blocking binding. Several studies have been made to ascertain whether such mechanisms exist. The results of one study indicated that propylthiouracil administration increased the T/S ratio by a mechanism in addition to the expected effect of blocking binding (Wollman and Scow, 1954b).

This paper presents the results of further studies made to detect evidence that a goitrogen might affect the T/S ratio by a mechanism in addition to that of blocking binding of radioiodide. These studies include, first, measurements of the T/S ratio and binding of radioiodide for graded doses of propylthiouracil which blocked binding to varying extents, and second, comparisons of the T/S ratio when binding was blocked by a variety of goitrogens, some of which have been suggested to block binding by mechanisms different from others.

MATERIALS AND METHODS

Male C3H mice, four to seven months of age, were used. They had been fed a pelleted diet of moderately low iodine content (Wollman, Scow, Wagner and Morris, 1953) for at least one week prior to the radioiodine studies.

Administration of goitrogens: All goitrogens[2] except 2-mercaptoimidazole were dis-

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[2] We are grateful to Dr. E. B. Astwood for samples of thiobarbital, 2-mercaptoimidazole and 2-mercaptothiazoline, to Dr. W. C. Schneider for the phloroglucinol and to the American Cyanamid Company for the sulfadiazine.

solved in 0.85% NaCl containing NaOH at a concentration of 0.04 N. The 2-mercaptoimidazole was dissolved in 0.85% NaCl. In general, the goitrogen was administered in 0.5 ml. of solution by subcutaneous injection within a few minutes after preparation of the solution. The 5 mg. dose of propylthiouracil and the 10 mg. dose of sulfadiazine, however, were each injected in 1 ml. of solution.

Administration of radioiodide: Aliquots of a stock radioiodide solution[3] were diluted with distilled water to a concentration of 20 microcuries per 0.50 ml., and 0.50 ml. was administered to each mouse by intraperitoneal injection one hour after the goitrogen injection.

Procedure at autopsy: Between one and two hours after the radioiodide injection each mouse was anesthetized with ether and a blood sample was obtained from the heart. The thyroid was removed immediately thereafter, cooled, weighed and then homogenized in 6 ml. of ice-cold distilled water containing 1 mg. of NaI. To test for completeness of blocking of binding and to measure the amount of radioiodide in the thyroid, the homogenates were routinely deproteinized with 0.2 ml. each of the $Ba(OH)_2$ and $ZnSO_4$ solutions of Somogyi.

Paper chromatography of supernates: The procedures for identifying the I^{131}-containing compounds in the supernates of homogenates of thyroids have been described previously (Wollman and Scow, 1954a).

I^{131} determinations: I^{131} in thyroid glands or in fractions thereof was measured by gamma counting, that in serum by beta or gamma counting as described previously (Wollman and Scow, 1954a).

RESULTS

Dependence of the T/S ratio on the dose of propylthiouracil: The T/S ratio was measured one hour after the radioiodide injection. The T/S ratio increased as the dose of propylthiouracil increased (Fig. 1). The increase in the T/S ratio was generally associated with a decrease in the amount of I^{131} bound in one hour. However, the rise in the T/S ratio on increasing the propylthiouracil dose from 2.5 to 5 mg. was not accompanied by a quantitatively important decrease in the amount of bound I^{131}. Instead, the increased T/S ratio was associated with a marked reduction in the serum radioiodide concentration.

To test if the higher dose of propylthiouracil had an effect on the rate of decrease of the blood radioiodide concentration, the blood radioiodide concentration was measured at various time intervals after injection of radioiodide. Serial tail vein blood samples of 0.03 ml. were obtained from four mice injected with 2.5 mg. of propylthiouracil and four with 5 mg. (Fig. 2). The blood radioiodide concentration was the same in the two groups at 15 minutes after radioiodide injection but at later time intervals it decreased significantly faster in the mice given 5 mg. of propylthiouracil.

Comparison of the T/S ratios obtained using various goitrogens: All of the goitrogens tested except pentothal were able to block organic binding

[3] The carrier-free radioiodide was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

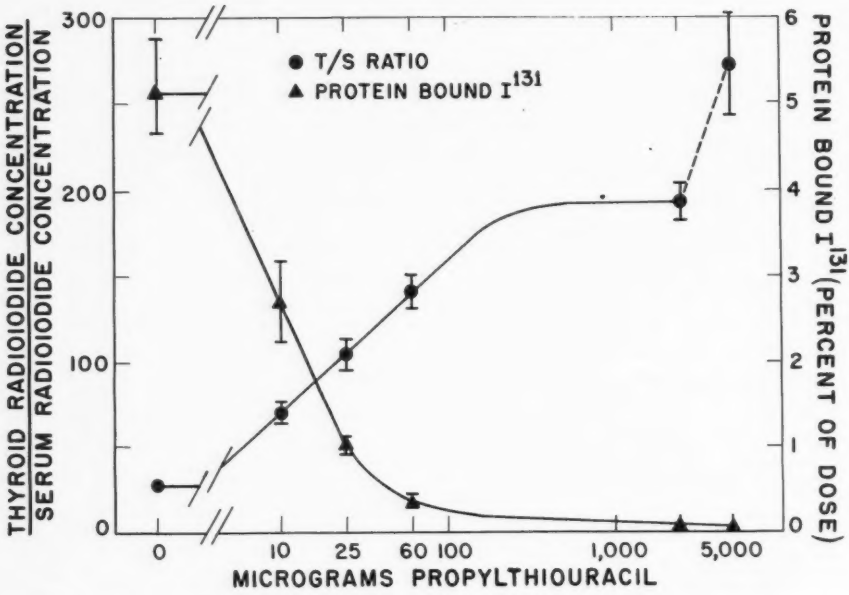

FIG. 1. The dependence of the T/S ratio and of the amount of radioiodine bound in one hour upon dose of propylthiouracil in C3H mice. The vertical line through each point represents the standard error.

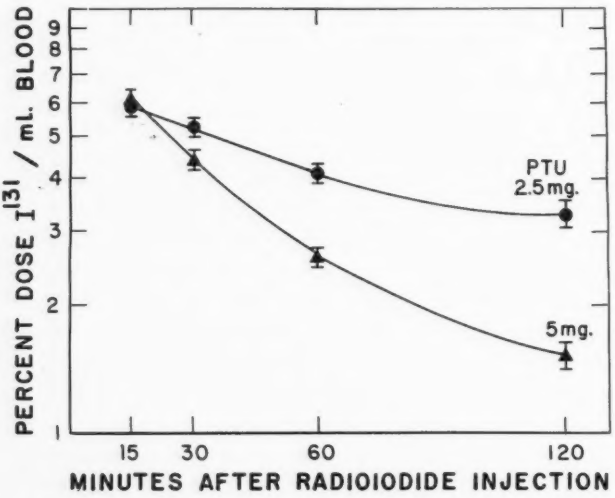

FIG. 2. The dependence of the concentration of radioiodide in the blood on the time interval after intraperitoneal injection of radioiodide in mice which had been given a single subcutaneous injection of 2.5 or 5 mg. of propylthiouracil.

of I^{131} almost completely (Table 1). Because of the incomplete blocking of binding with pentothal, the T/S ratios using this substance may not be compared with those for the other goitrogens. The T/S ratio was nearly the same for most of the goitrogens. However, for phloroglucinol and 2-mercaptothiazoline the T/S ratio was significantly lower. The 2-mercaptothiazoline also differed from the other goitrogens in that the serum radioiodide concentration was significantly less than that for other goitrogens.

The nature of the I^{131} in the supernates of thyroid homogenates deproteinized by $Ba(OH)_2$ and $ZnSO_4$ was examined in mice in which binding

TABLE 1. A COMPARISON OF THE RATIO OF THYROID RADIOIODIDE CONCENTRATION TO SERUM RADIOIODIDE CONCENTRATION (T/S) USING SINGLE INJECTIONS OF VARIOUS GOITROGENS TO BLOCK ORGANIC BINDING

Goitrogen	Dose, mg.	No. mice	Mouse weight, gm.	Thyroid weight, mg.	T/S	Thyroid I^{131} sup.[4] / Thyroid I^{131} sed.	Per cent dose I^{131} per gm. serum[3]	Minutes between I^{131} injection and autopsy
Pentothal	2	4	29±3[1]	3.0±0.2[1]	80± 3[2]	1.17	5.2 ±0.2[2]	70
Thiobarbital	5	8	32±2	3.0±0.4	153±15	16	4.9 ±0.3	50 to 80
2-Mercaptothiazoline	5	8	29±2	2.7±0.5	124± 5	19	3.1 ±0.2	110
2-Mercaptoimidazole	5	9	29±2	2.5±0.5	254±26	15	3.9 ±0.1	80
Thiouracil	2.5	4	31±2	2.8±0.5	165± 7	17	3.77±0.15	120
Propylthiouracil	1	5	32±1	2.5±0.1	161±17	16	5.3 ±0.2	70
	2.5	18	29±3	2.6±0.3	178± 7	16	3.7 ±0.1	65 to 110
Sulfadiazine	5	4	32±1	3.1±0.1	164±11	8	3.8 ±0.4	85
	10	9	28±3	2.3±0.5	175±19	11	4.3 ±0.1	60
Phloroglucinol	5	15	32±3	2.5±0.5	132± 8	20	4.8 ±0.3	60 to 100

[1] Standard deviation.
[2] Standard error of the mean.
[3] Corrected to 30 gm. body weight.
[4] Sup.=supernate; sed.=sediment.

was blocked by a single injection of propylthiouracil (5), sulfadiazine (1), phoroglucinol (5) or 2-mercaptothiazoline (1) (the figure in parenthesis represents the number of mice studied). Solvents used were ethanol/ammonium hydroxide and n-butanol/acetic acid/water. Results obtained were quantitatively as previously reported for propylthiouracil (Wollman and Scow, 1954a). In ethanol/ammonium hydroxide almost all the I^{131} put on the paper was recovered in the iodide spot. In no case was any evidence obtained of the presence of I^{131} in a compound other than iodide in amounts of more than a few per cent of the I^{131} in the iodide spot.

DISCUSSION

It was shown in a previous paper (Wollman and Scow, 1954b) that if the concentration of stable thyroid iodide is high enough to block binding of I^{131}, administration of propylthiouracil increases the T/S ratio by a mechanism different from that associated with the elevation expected to accompany a block in the binding of iodide. It appeared desirable to determine whether the same mechanism operated at physiological levels

of stable iodide. The criterion for the detection of this mechanism was the observation of a change in the T/S ratio on altering the propylthiouracil dose without a corresponding change in the amount of I^{131} bound in the thyroid. As the dose increased from 0 to 2.50 mg. of propylthiouracil, the increase in the T/S ratio was always associated with a decrease in the amount of I^{131} bound. Therefore no evidence for the mechanism was obtained in this dose range. However, this test cannot be considered conclusive because a mechanism might exist which causes an increase in the T/S ratio with increasing dose of propylthiouracil in the same dose range as that in which progressive blocking of the binding mechanism occurs. In order to test for such a mechanism in this dose range it will be necessary to compare the observed relation between the T/S ratio and the binding rate constant with that predicted on the basis of the assumption that propylthiouracil acts only by blocking binding (Wollman, 1954). Deviations of the experimental results from the theory might indicate the existence of this mechanism.

A different mechanism was detected in the propylthiouracil dose range between 2.5 and 5 mg. A dose of 5 mg. of propylthiouracil was associated with a significantly higher T/S ratio than a dose of 2.5 mg. or less (Fig. 1) with no significant change in the amount of I^{131} bound. The increase in the T/S ratio in this dose range seems to be related to the abnormally high rate of decrease of the blood radioiodide concentration in mice given 5 mg. of propylthiouracil. A recently suggested model of the thyroid (Wollman, 1954) yields the prediction that the T/S ratio is expected to vary inversely as $1-\tau_e/\tau$ where τ_e is the reciprocal of the rate constant for equilibration of radioiodide between thyroid gland and blood and τ is the reciprocal of the rate constant for the decrease of the radioiodide concentration in the blood. This relation predicts that the T/S ratio in mice given 2.5 or 5 mg. of propylthiouracil is expected to be 1.20 or 1.48 times as high as in mice in which the blood radioiodide concentration remains constant, respectively. In this calculation τ_e is taken as 14 minutes (Wollman and Scow, 1953) and τ (the mean life) at one hour after radioiodide injection is taken to be 84 and 46 minutes (Fig. 2) for mice given 2.5 and 5 mg. of propylthiouracil, respectively. Hence the T/S ratio using 5 mg. of propylthiouracil is expected to be 1.23 times as high as when using 2.5 mg. This accounts for the observed difference in the T/S ratios within the experimental error in the data.[4]

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[4] This effect appears to have been the cause of a systematic error in estimates of the T/S ratio in a recent paper (Wollman and Scow, 1954a). T/S ratios were measured using 2.5 mg. of propylthiouracil and this resulted in values that were too high for low serum iodide concentrations. As a consequence the estimates of K_M reported there for mice are too low. Recent studies using 1.0 mg. of propylthiouracil to block binding give a K_M of approximately 0.5 mg. per 100 ml. for normal mice in good agreement with values for the rat.

Since the rate of change of the blood radioiodide concentration decreases with time at sufficiently long time intervals after injection of I^{131} (Fig. 2) the elevation of the T/S ratio due to the preceding effect might be expected to decrease at sufficiently long time intervals. The curve describing the dependence of the T/S ratio on time interval after radioiodide injection may therefore be expected to have a maximum. This effect may have been observed in an earlier study on the equilibration of radioiodide between thyroid gland and blood (Fig. 2 of the paper by Wollman and Scow, 1953) in which the T/S ratio at one hour after radioiodide injection was approximately 20% higher than at later time intervals.

The T/S ratio following single injections of all goitrogens was much higher than when binding was permitted. The relatively constant value of the T/S ratio for a variety of goitrogens of significantly different structures is in agreement with the hypothesis that single doses of many of the goitrogens tested influence the T/S ratio largely by blocking binding. Two of the goitrogens, 2-mercaptothiazoline and phloroglucinol, may affect the T/S ratio to a small extent by some additional mechanism as well (Table 1). For example, the T/S ratio using the 2-mercaptothiazoline was significantly lower than that with most other goitrogens. The observed value may be actually larger than the correct value, for when using this goitrogen, the serum radioiodide concentrations were very low. The T/S ratio may be elevated by the same mechanism as that discussed above for 5 mg. of propylthiouracil, namely, by a rapid fall in blood radioiodide level.

Fawcett and Kirkwood (1953) have suggested that phloroglucinol prevents the formation of protein-bound iodine by competing with the protein for iodine, and by being iodinated instead of the protein. In such a circumstance the iodination might be expected to occur as rapidly as if the protein were being iodinated. The T/S ratio would then be expected to be low as it is in the unblocked gland (Fig. 1). The high T/S ratio observed in the present experiments might then be explained by the presence of a high concentration of iodinated phloroglucinol. However, the present studies using chromatography indicate that, when blocked by phloroglucinol, the thyroid contains I^{131} almost entirely in the form of iodide. This suggests that the iodination of phloroglucinol must be a quantitatively negligible process in the mouse; and that the block in protein iodination by phloroglucinol is probably by some mechanism other than that suggested by Fawcett and Kirkwood (1953).

SUMMARY

The ratio of the radioiodide concentration in the thyroid gland and serum (T/S ratio) was studied in young male mice. As little as 10 μg. of propylthiouracil caused a partial inhibition of the binding of radioiodide by the thyroid; this was associated with an increase in the T/S ratio.

Between 10 and 60 μg. the T/S ratio progressively increased whereas the binding of radioiodide decreased. Between 60 and 2500 μg. the binding process was almost completely blocked and the T/S ratio remained almost constant. With 5 mg., however, the T/S ratio was significantly elevated above that with 2.5 mg. of propylthiouracil. This was correlated with an unusually high rate of decrease of the blood radioiodide concentration following the larger dose of propylthiouracil.

The T/S ratio was also measured using thiouracil, 2-mercaptoimidazole, thiobarbital, 2-mercaptothiazoline, sulfadiazine and phloroglucinol to block binding. The T/S ratio was relatively constant and independent of the goitrogen. The I^{131} in the thyroids blocked by the last three goitrogens and by propylthiouracil was almost entirely in the form of iodide.

Acknowledgment

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REFERENCES

Fawcett, D. M. and S. Kirkwood: *J. Biol. Chem.* **204:** 787. 1953.
Wollman, S. H.: *Endocrinology* **54:** 35. 1954.
Wollman, S. H. and R. O. Scow: *Endocrinology* **53:** 332. 1953.
Wollman, S. H. and R. O. Scow: *Endocrinology* **55:** 828. 1954a.
Wollman, S. H. and R. O. Scow: *Endocrinology* **55:** 837. 1954b.
Wollman, S. H., R. O. Scow, B. Wagner and H. P. Morris: *J. Nat. Cancer Inst.* **13:** 785. 1953.

PHOSPHORUS METABOLISM OF THE ADRENAL GLAND:

EFFECT OF HYPOPHYSECTOMY AND ADMINISTRATION OF ACTH ON THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO THE RNA NUCLEOTIDES[1]

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THE current interest in the role of ribonucleic acid (RNA) in cellular function made it desirable to compare the phosphorus metabolism of the RNA in a tissue during low functional activity with that of the same tissue during high functional activity—for instance, the adrenal gland of the hypophysectomized rat compared with the adrenal gland of rats treated with ACTH.

It is known that removal of the pituitary gland causes a decrease in the incorporation of inorganic phosphate labeled with P^{32} into the total acid-soluble phosphorus fraction (Reiss and Halkerston, 1950), the inorganic phosphate (Gemzell and Samuels, 1950; Riedel, Logan, DeLuca and Rossiter, 1954) and the lipid phosphorus (Riedel, Logan and Rossiter, 1954) of the adrenal gland. In each instance the rate of incorporation is largely restored to normal by a single injection of ACTH.

In the experiments described in this paper, the incorporation of P^{32} into the RNA of the adrenal gland was studied. A preliminary report of these experiments has already appeared (Logan, and Heagy, 1954).

METHODS

Animals

The animals were male rats of the Sprague-Dawley strain. Hypophysectomized rats, supplied by the Hormone Assay Laboratory, Inc., Chicago, Illinois, were used for the experiment two days after operation. The adrenal glands of hypophysectomized animals displayed the typical atrophy associated with hypophysectomy. Macroscopic and microscopic examination of the pituitary region of selected animals showed no evidence of pituitary tissue remaining. The animals were fasted 12 to 14 hours before killing.

ACTH Preparation

The ACTH was obtained from Connaught Medical Research Laboratories, Toronto, Ontario. It was a white powder, completely soluble in isotonic saline. The preparation had been standardized in terms of the International Standard.

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Experimental Procedure

Test animals received a single intraperitoneal injection of ACTH, 4 mg. per 100 gm. body weight, in isotonic saline. Control animals received an equivalent volume of isotonic saline. Four hours later all the animals received 200 μc. P^{32}*, regardless of body weight, by intraperitoneal injection.

The animals were killed 16 hours after the P^{32} injection, i.e., 20 hours after the injection of ACTH or saline. Under nembutal anaesthesia, a midline incision was made in the abdomen at the height of the diaphragm. A sample of blood was withdrawn from the inferior vena cava into a 2 cc. syringe containing a small quantity of heparin. The blood was transferred, with care to prevent hemolysis, to a centrifuge tube that was kept in an ice-bath. The adrenal glands were excised as rapidly as possible, blotted to remove excess blood, trimmed carefully to remove adhering adipose and connective tissue, and frozen rapidly by plunging into liquid nitrogen. The blood sample then was centrifuged in the cold room.

Analytical and Counting Methods

The adrenals from six rats were pooled to provide sufficient tissue and weighed in the frozen state. They were homogenized in ice-cold 10% trichloroacetic acid using a homogenizer of the Potter-Elvehjem type. The concentration and the specific activity of the inorganic P of the trichloroacetic acid extracts of a pooled sample of plasma and the pooled adrenals were determined by the method of Ernster, Zetterström and Lindberg (1950). The tissue residue was washed four times with 10% trichloroacetic acid in the cold to remove the last traces of acid-soluble radioactivity. The residue was then extracted successively with ethanol (2 portions), ethanol-ether 3:1 (5 portions heated to boiling) and ether to remove lipid phosphorus. The residue remaining after removal of the lipid P was extracted three times with 10% sodium chloride at 100° C. to obtain the nucleic acids. The nucleates were precipitated with two volumes absolute ethanol, washed twice with 5% trichloroacetic acid and dried with ethanol and ether. Hydrolysis of the nucleic acids was carried out in 0.3 N KOH at 37° C. and the resulting acid-soluble RNA nucleotides were separated by ionophoresis on paper according to the method of Davidson and Smellie (1952a). After elution, the nucleotides were ashed with 60% perchloric acid by the method of King (1932) and the concentration and specific activity of the phosphorus determined as described for the inorganic P.

In the method of Ernster *et al.* (1950) both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue colour complex was read with a Beckman Model B Spectrophotometer at 730 mμ and the radioactivity due to the P^{32} in the samples was determined on the same coloured solution using an M-6 liquid counter (20th Century Electronics). The counting rate was corrected for background and decay. The specific activities for each animal were adjusted to a standard injection of 200 μc. per 200 gm. body weight, based on a simulated P^{32} standard (Tracerlab. Inc., Boston).

RESULTS

Plasma Inorganic Phosphate

The specific activity of the inorganic phosphate of the plasma 16 hours after the injection of P^{32} was not changed significantly by hypophysectomy (2 days after the operation) or by the administration of ACTH (Table 1).

* Supplied in the form of $H_3P^{32}O_4$ by Commercial Products Division, Atomic Energy of Canada, Limited.

This finding is in agreement with the previous report of Riedel *et al.* (1954b). However, at shorter time intervals after the P^{32} injection (Gemzell and Samuels, 1950; Riedel *et al.*, 1954a), and at longer times after hypophysectomy (Geschwind, Li and Evans, 1951; Riedel and Rossiter, 1954; Riedel *et al.* 1954b), there is an increase in the specific activity of the inorganic phosphorus in the plasma of hypophysectomized rats. There is evidence that this change is the result of a deficiency of growth hormone (Geschwind *et al.*, 1951; Riedel *et al.*, 1954a).

Adrenal Inorganic Phosphate

Table 1 shows that hypophysectomy caused a decrease in the specific activity of the adrenal inorganic phosphate and that this decrease was prevented by a single injection of ACTH, thus confirming the finding of

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND THE ADMINISTRATION OF ACTH ON THE SPECIFIC ACTIVITY OF THE INORGANIC PHOSPHATE OF THE PLASMA AND THE RELATIVE SPECIFIC ACTIVITY OF THE INORGANIC PHOSPHATE OF THE ADRENAL GLAND OF THE RAT

Time after ACTH, 20 hr. Time after P^{32}, 16 hr. Mean ±S.E. mean

Group	No. determinations	Plasma		Adrenal	
		Cts./min./ μg. P[3]	P	Specific activity relative to plasma $\times 10^2$	P
Normal	14	95 ±4.2		79.0 ±1.2	
Hypophysectomized	8	104 ±2.3	0.1–0.2[1]	70.6 ±3.4	<0.02[1]
Normal + ACTH	6	97 ±8.9	0.8–0.9[2]	99.9 ±3.7	<0.001[2]
Hypophysectomized + ACTH	8	101 ±2.8	0.7–0.8[2]	82.7 ±2.2	<0.01[2]

[1] P value for hypophysectomized rats compared with normals.
[2] P value for rats receiving ACTH compared with similar animals receiving saline.
[3] With the counting equipment used, the injected dose was 4.29×10^7 cts./min. for a rat of body weight 200 gm.

Gemzell and Samuels (1950) and Riedel *et al.* (1954a). In addition, a significant increase in the relative specific activity of the adrenal inorganic phosphate was demonstrated when ACTH was injected into normal animals. A trend in this direction was noted by Gemzell and Samuels (1950) and Riedel *et al.* (1954a), but the figures did not attain the conventional level of statistical significance. In the present study the pooling of the adrenal glands (6 rats for each determination) has minimized the effect of animal-to-animal variation and so rendered statistical significance to the observation.

RNA Nucleotide Phosphorus

Table 2 shows the effect of hypophysectomy and of the administration of ACTH on the incorporation of P^{32} into the four RNA nucleotides of the adrenal gland. In the hypophysectomized animals, the specific activity of each of the four nucleotides, relative to the specific activity of the ad-

renal inorganic phosphate, was decreased. The administration of ACTH to the normal animals increased the mean relative specific activity of each of the four nucleotides, but in no instance was the increase significant statistically. On the other hand, a significantly increased incorporation into each of the four nucleotides was observed when ACTH was given to the hypophysectomized animals. Thus a single injection of ACTH was able to reverse the changes brought about by hypophysectomy. These findings confirm previous work reported from this laboratory (Riedel and DeLuca, 1953), in which the specific activity of the total RNA, isolated by the method of Hammarsten (1947), was studied.

DISCUSSION

The figures reported in Table 2 make it clear that the labeled phosphate is incorporated into the nucleotides of the RNA of the adrenal gland and that, as found for liver tissue *in vivo* (Volkin and Carter, 1951; Davidson

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND THE ADMINISTRATION OF ACTH ON THE RELATIVE SPECIFIC ACTIVITY OF THE RNA NUCLEOTIDES OF THE ADRENAL GLAND OF THE RAT

Time after ACTH, 20 hr. Time after P^{32}, 16 hr. Mean ±S.E. mean

Group	Specific activity relative to adrenal inorganic P×10²								
	Adenylic acid	*P*	Guanylic acid	*P*	Uridylic acid	*P*	Cytidylic acid	*P*	Average
Normal	26.6±1.7 (11)[1]		22.4±1.9 (12)		25.7±1.8 (11)		22.5±1.3 (10)		24.3
Hypophysectomized	20.4±1.0 (5)	<0.05[2]	16.2±1.3 (6)	<0.05[2]	19.5±2.0 (5)	<0.1[2]	18.1±1.6 (5)	<0.1[2]	18.6
Normal+ACTH	29.4±1.5 (5)	>0.3[3]	25.3±0.6 (5)	>0.3[3]	28.6±1.2 (5)	>0.3[3]	26.1±0.9 (5)	>0.05[3]	27.4
Hypophysectomized +ACTH	25.6±1.7 (4)	<0.05[3]	21.9±0.4 (6)	<0.01[3]	26.4±1.0 (4)	<0.05[3]	24.4±1.8 (4)	<0.05[3]	24.6

[1] No. determinations.
[2] *P* value for hypophysectomized rats compared with normals.
[3] *P* value for rats receiving ACTH compared with similar animals receiving saline.

and Smellie, 1952b) and brain slices *in vitro* (DeLuca, Rossiter and Strickland, 1953), there is a greater incorporation into adenylic acid and uridylic acid than into guanylic acid and cytidylic acid. In experiments such as these, isolation of the nucleotides is necessary, since the tissue RNA phosphorus of both liver (Davidson, Frazer and Hutchison, 1951) and brain (DeLuca *et al.*, 1953), when separated by the usual procedures, contains a number of high-activity non-nucleotide phosphorus-containing compounds. The nucleotides studied were mixtures of the *a* and *b* isomers (Carter and Cohn, 1949), now known to be the 2′- and 3′- phosphoribosides respectively (Brown *et al.*, 1953; Khym and Cohn, 1954).

The mechanism of the change in the phosphorus metabolism of the adrenal gland after hypophysectomy is of some interest. The inorganic phosphate of the adrenal, like that of any other tissue, is partly extracellular (plasma and interstitial fluid) and partly cellular. The difficulties of determining the specific activity of the cellular inorganic phosphate are

well known (Ennor and Rosenberg, 1954; Riedel and Rossiter, 1954). It is widely believed that after an injection of inorganic P^{32} the isotope rapidly equilibrates with the inorganic phosphate of the extracellular space, but that passage of the isotope through the cell membrane into the cellular fluid is much slower (Hahn and Hevesy, 1941; Hevesy, 1948). However, the validity of this assumption has been questioned for rabbit muscle (Ennor and Rosenberg, 1954).

Riedel and Rossiter (1954) concluded that there was a genuine reduction in the specific activity of the cellular inorganic phosphate of the adrenals of hypophysectomized rats, resulting from a decrease in the rate at which inorganic P^{32} passed through the cell membrane, i.e. from the extracellular to the cellular portion of the gland. In addition, Riedel *et al.* (1954b) showed that in the adrenal of the hypophysectomized rat there was a decrease in the rate of formation of lipid phosphorus from cellular inorganic phosphate. The finding that the specific activity of each of the adrenal RNA nucleotides was decreased relative to that of the tissue inorganic phosphate suggests that for RNA a similar situation might attain.

Riedel *et al.* (1954b), after reviewing the evidence, concluded that there were at least two changes in the phosphorus metabolism of the adrenal glands of hypophysectomized rats: (a) slowing of the passage of inorganic phosphate from the extracellular to the intracellular portion of the gland and (b) a slowing in the formation of lipid phosphorus from cellular inorganic phosphate. The experiments reported here indicate that there is also a slowing in the formation of nucleotide phosphorus from cellular inorganic phosphate and that this slowing can be overcome by the administration of ACTH. These findings for RNA provide additional support for the suggestion of Riedel *et al.* (1954b) that the changes in incorporation of P^{32} into both the cellular inorganic and organic phosphorus fractions of the adrenal gland following hypophysectomy or following administration of ACTH may reflect changes in the over-all metabolism of the adrenal gland, rather than the specific effects on the tissue constituent under study. The primary site of the defect that follows hypophysectomy and the primary site of action of ACTH are not known.

SUMMARY AND CONCLUSIONS

The specific activity of the phosphorus of each of the four RNA nucleotides of the adrenal gland of hypophysectomized rats, relative to that of the adrenal inorganic phosphate, was decreased 16 hours after the administration of inorganic P^{32}. These decreases were eliminated by a single injection of ACTH 4 hours before the administration of the P^{32}.

It is concluded that in the adrenal gland of hypophysectomized rats there is: (a) a decrease in the rate at which inorganic P^{32} passes from the extracellular to the cellular portion of the gland and (b) a decrease in the rate of formation of organic phosphorus compounds (e.g. lipid phos-

phorus, RNA nucleotides) from the cellular inorganic phosphate. It is suggested that these changes, and those in the opposite direction that follow the administration of ACTH, may reflect changes in the over-all energy metabolism of the adrenal gland, rather than specific effects on the constituent under study.

REFERENCES

BROWN, D. M., G. B. FASMAN, D. I. MAGRATH, A. R. TODD, W. COCHRAN AND M. M. WOOLFSON: *Nature* **172:** 1184. 1953.

CARTER, C. E. AND W. E. COHN: *Federation Proc.* **8:** 190. 1949.

DAVIDSON, J. N., S. C. FRAZER AND W. C. HUTCHISON: *Biochem. J.* **49:** 311, 1951.

DAVIDSON, J. N. AND R. M. S. SMELLIE: *Biochem. J.* **52:** 594. 1952a.

DAVIDSON, J. N. AND R. M. S. SMELLIE: *Biochem. J.* **52:** 599. 1952b.

DELUCA, H. A., R. J. ROSSITER AND K. P. STRICKLAND: *Biochem. J.* **55:** 193. 1953.

ENNOR, A. H. AND H. ROSENBERG: *Biochem. J.* **56:** 302. 1954.

ERNSTER, L., R. ZETTERSTRÄM AND O. LINDBERG: *Acta Chem. Scand.* **4:** 942. 1950.

GEMZELL, C. A. AND L. T. SAMUELS: *Endocrinology* **47:** 48. 1950.

GESCHWIND, I. I., C. H. LI AND H. M. EVANS: *Arch. Biochem.* **31:** 168. 1951.

HAHN, L. AND G. HEVESY: *Acta Physiol. Scand.* **1:** 347. 1941.

HAMMARSTEN, E.: *Acta Med. Scand.* Supp. **196:** 634. 1947.

HEVESY, G.: *Radioactive Indicators.* Interscience Publishers Inc., N. Y. 1948.

KHYM, J. X. AND W. E. COHN: *J. Am. Chem. Soc.* **76:** 1818. 1954.

KING, E. J.: *Biochem. J.* **26:** 292. 1932.

LOGAN, J. E. AND F. C. HEAGY: *Federation Proc.* **13:** 254, 1954.

REISS, M. AND J. M. HALKERSTON: *J. Endocrinol.* **6:** 369. 1950.

RIEDEL, B. E. AND H. A. DELUCA: *Federation Proc.* **12:** 258. 1953.

RIEDEL, B. E., J. E. LOGAN, H. A. DELUCA AND R. J. ROSSITER: *Canad. J. Biochem. Physiol.* **32:** 251. 1954a

RIEDEL, B. E., J. E. LOGAN AND R. J. ROSSITER: *Endocrinology* **55:** 219. 1954b.

RIEDEL, B. E. AND R. J. ROSSITER: *Canad. J. Biochem. Physiol.* **32:** 261. 1954.

VOLKIN, E. AND C. E. CARTER: *J. Am. Chem. Soc.* **73:** 1519. 1951.

THE EFFECTS OF LOCALLY APPLIED ESTROGEN ON THE PUBIC SYMPHYSIS AND KNEE JOINT IN CASTRATED MICE[1]

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FOLLOWING puberty a sexual dimorphism of the pelvis develops in mice. At this time the medial ends of the pubic bones at the symphysis in the female undergo partial resorption (Gardner, 1936). During the first pregnancy the ventromedial part of the pubes is resorbed further and an interpubic ligament replaces the symphysial cartilage (Hall, 1947; Crelin, 1954a, 1954b). Similar changes occur in intact females and gonadectomized mice when estrogens are injected (Gardner, 1936; Crelin, 1954b, 1954c). Both the normal and induced pubic bone resorption follow a similar pattern. The cranio-dorsal surfaces of the medial ends of the pubes are affected first. The resorption then continues along the medial margin of each pubic bone in a caudo-lateral direction (Hall, 1950). As this process occurs in estrogen-injected mice all the other bones of the body undergo increased calcification (Gardner and Pfeiffer, 1938, 1943). The injected estrogen also induces a breakdown of the symphysial cartilage and its replacement by an interpubic ligament. While this is occurring no other articular cartilage of the body undergoes such a marked change. Why the interpubic tissue and pubes respond so specifically to estrogen and why the hormone produces such a consistent pattern of pubic bone resorption is unknown (Hall, 1950, 1951; Crelin, 1954c).

The influence of estrogen on the pubes and pubic symphysis has been observed only following its systemic injection. In the present experiment estrogen was applied locally to the bones and soft tissues at the pubic symphysis and knee joint.

MATERIALS AND METHODS

Sixty-eight mice of the Brown-belt strain were castrated when 3 weeks old. Two weeks after castration they were arranged into 3 groups and received the following treatment:

Group I 13 ♂, 16 ♀ *Untreated Controls.* These animals received no treatment.

Group II 2 ♂, 3 ♀ *Glass-treated Controls.* The ventral surface of the pubic symphysis was exposed in each mouse by making a midline incision in the abdominal skin. The symphysis was then viewed under a stereoscopic microscope and a small perforation was

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made in the linea alba at its cranial border. Using iris forceps a small amount of fine glass particles was passed through the perforation and placed between the urinary bladder and the symphysis. The particles were then in direct contact with the dorsal surface of the symphysis. Following this, a small incision was made on the medial side of the left knee, through which each animal received a small implant of glass particles under the patellar ligament. These two implantations were made in the same places where the mice of Group III received fine estrone particles; thus the glass-treated animals served as controls in determining what tissue changes might result from mechanical irritation which the estrone particles might have produced.

Group III 18 ♂, 16 ♀ *Estrogen-treated Mice.* Powdered estrone was dissolved in ether and alcohol. During slow evaporation the estrone precipitated out in the form of crystals and thin flat sheets. The sheets were broken into tiny particles with a sharp pointed forceps. An implant of 1–2mg. of the estrone particles was placed in each animal at the dorsal surface of the symphysis and under the left patellar ligament in the same manner as the glass-treated controls received the glass implants.

Thirty days following the implantation date all of the animals were sacrificed by digital separation of the atlanto-occipital joint. The symphysis was exposed and its flexibility determined in situ by grasping the innominate bones with forceps and moving them in opposite directions. The symphysis was considered flexible only when the range of movement was marked. The narrowest point of separation between the innominate bones at the symphysis was measured directly with a caliper by a method previously reported (Crelin, 1954b). The gross appearance of the uterus and urinary bladder was noted. The innominate bones, excluding most of the ilia, and the intact symphysis were then removed en bloc and preserved. Following skinning of the left leg the lower half of the thigh and the upper half of the lower leg were transected. The excised block of tissue containing the knee joint was preserved. The right knee and both humeri of a number of estrogen-treated mice were also preserved for study so that the degree of systemic estrogen effect could be determined. A few symphyses and knee joints, which were considered to be typical of each group were fixed in 10% formalin and prepared as whole mounts. The soft tissue of the mounts was macerated in KOH, cleared in glycerin and the bones were stained with alizarin-red S. The remaining tissues were fixed in Bouin's fluid, decalcified with Haug's solution, embedded in paraffin and serially-sectioned. Two staining procedures were used: 1. Foot's modification of the del Rio Hortega silver carbonate stain to demonstrate reticulum fibers; 2. toluidine blue to demonstrate the presence of hyaline cartilage which stains metachromatically.

RESULTS

Group I 13 ♂,16 ♀ Untreated Controls. At autopsy the pubic symphysis in each mouse was not flexible nor was there a measurable interpubic gap. Also, the urinary bladder was normal and undistended. The uterus in the female was atrophic. Whole mounts of the pelves revealed that although these 9-week-old animals were castrated about 3 weeks before puberty a definite sexual dimorphism developed. The pelvic dimorphism differed from the normal only in the lack of bone resorption along the cranio-dorsal and medial surfaces of the pubes in the female. The male pelvic bones, particularly the pubes, were thicker and broader than those of the females. The medial surfaces of the pubic bones which are closely approximated and parallel at the symphysis were more extensive in the male. In the female

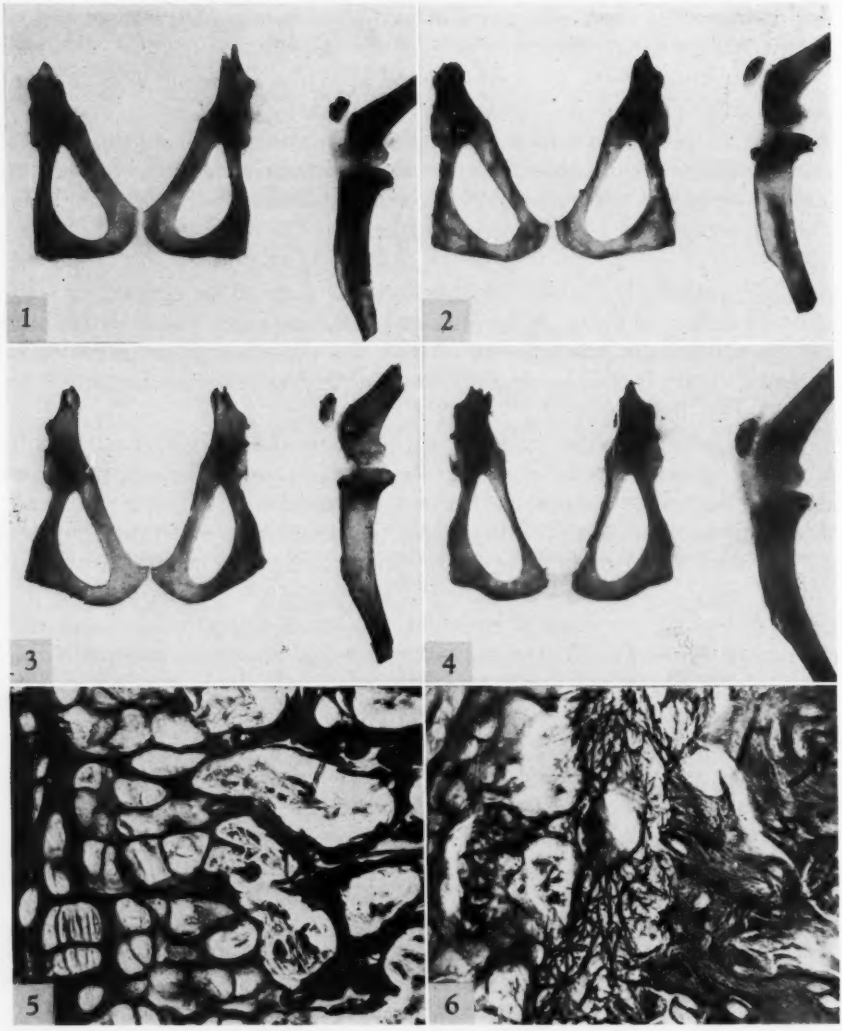
the ischio-pubic angle was more obtuse and the symphysis more posteriorly placed (Figs. 1, 3).

Microscopically the interpubic tissue appeared the same in both sexes. The medial ends of the pubes were covered by thick articular caps of hyaline cartilage which were continuous with a thin midline strip of fibrocartilage. The medial ends of the pubes consisted chiefly of osseous trabeculae covered by osteoblasts. Numerous capillaries were present where the trabeculae abutted the rowed cartilage at the osseo-chondral junction (Fig. 5). The greater portion of the marrow of the pubes and ischia contained hemopoietic tissue. The symphysial fibrocartilage stained by toluidine blue was slightly metachromatic (bluish-purple) and the hyaline cartilage intensely metachromatic. The Foot-Hortega stain revealed no reticular fibers in the interpubic tissue or in the marrow cavities of the pubes (Fig. 5).

The adjacent articular surfaces of the femur and tibia were covered by hyaline cartilage supported by a thin layer of bone. The epiphyses and patella had well-developed marrow cavities containing hemopoietic tissue. A narrow layer of bone lined the epiphysial cartilage and thin bony trabeculae extended from this layer through the marrow cavity to the one supporting the articular cartilage. The epiphysial plate consisted of a thin layer of hyaline cartilage adjacent to a layer of columnar cartilage cells averaging 10 cells thick: the range being 6–15. This layer merged with a layer of hypertrophied cartilage averaging 3 cells thick, the range being 2–5, which was next to the zone of provisional calcification in the metaphysis. Slender spicules of newly-calcified bone, oriented roughly parallel to the long axis of the femur and tibia, were present in moderate amount in the metaphysis. Some of them contained remnants of calcified cartilage and all were lined with osteoblasts. The marrow of the metaphyses contained hemopoietic tissue and the region of newly calcified cartilage was richly supplied with capillaries (Figs. 7, 9). Toluidine blue stained the knee cartilage the same as it did the symphysial cartilage and the Foot-Hortega method did not show any demonstrable reticular fibers in the sections of the knee (Fig. 11).

Group II 2 ♂,3 ♀ Glass-treated Controls. At autopsy the glass particles were still in place. They were carefully removed so that the tissue could be sectioned. Except for a slight increase of connective tissue in the immediate vicinity of the implant the findings were the same as those described for the untreated controls.

Group III 18 ♂,16 ♀ Estrogen-treated Mice. At autopsy particles of estrone were found at both implantation sites in the majority of animals. They were carefully removed. The symphysis was flexible in each mouse and an interpubic gap was measurable. The gap averaged 0.3 mm. in the males: the range being 0.25–0.5 mm. In the females it averaged 0.6 mm.:



EXPLANATION OF FIGURES 1–6

FIG. 1. Bones of the left knee (lateral view) and innominate bones (anterior view), excluding most of the ilia, from a castrated, untreated male. (×3)

FIG. 2. Bones of the left knee (lateral view) and innominate bones (anterior view), excluding most of the ilia, from a castrated, estrogen-treated male. The cranio-dorsal and medial surfaces of the pubic bones have undergone some resorption. Before fixation the interpubic gap measured 0.5 mm. Grossly, the patella and portions shown of the femur and tibia appear normal. (×3)

FIG. 3. Bones of the left knee (lateral view) and innominate bones (anterior view), excluding most of the ilia, from an ovariectomized, untreated female. (×3)

FIG. 4. Bones of the left knee (lateral view) and innominate bones (anterior view),

the range being 0.25–1.5 mm. The urinary bladders were greatly distended in 15 males and 3 females. One male and 8 females had empty bladders whose walls were hypertrophied. All uteri were greatly distended with a serous fluid which filled the lumen.

Bone resorption occurred along the cranio-dorsal surface and medial margin of the pubes of each animal, being most pronounced in the females (Figs. 2, 4). While the pubic bones were resorbed at the symphysis endosteal bone was formed lateral to the portions being resorbed so that the medial-most portion of the pubic marrow cavity was almost obliterated. Osteoclasts were present along the surfaces undergoing resorption. The articular caps of hyaline cartilage were variably resorbed and the symphysial fibrocartilage replaced by dense collagenous connective tissue. The interpubic tissue of 3 males and 4 females consisted almost entirely of dense ligamentous tissue connected to the pubic bones by networks of fine reticular fibers (Fig. 6). The ligament and proliferating connective tissue replaced the articular caps and medial part of the pubic bones. Small islands of unresorbed articular cartilage stained metachromatically with toluidine blue in contrast with the adjacent orthochromatically-stained connective tissue and bone matrix. The interpubic tissue of 3 males and 5 females consisted of both cartilage and connective tissue. In the remaining 7 males and 2 females slight changes occurred for although the articular caps were thinner the interpubic tissue consisted almost entirely of fibrocartilage. In all but 4 mice the amount of symphysial fibrocartilage replaced by connective tissue was proportional to the interpubic gap. Reticular fibers were not noted in the marrow of the pubes.

The whole mounts of the knee were comparable to those of the controls (Figs. 2, 4). Microscopically the effects of the estrone induced similar

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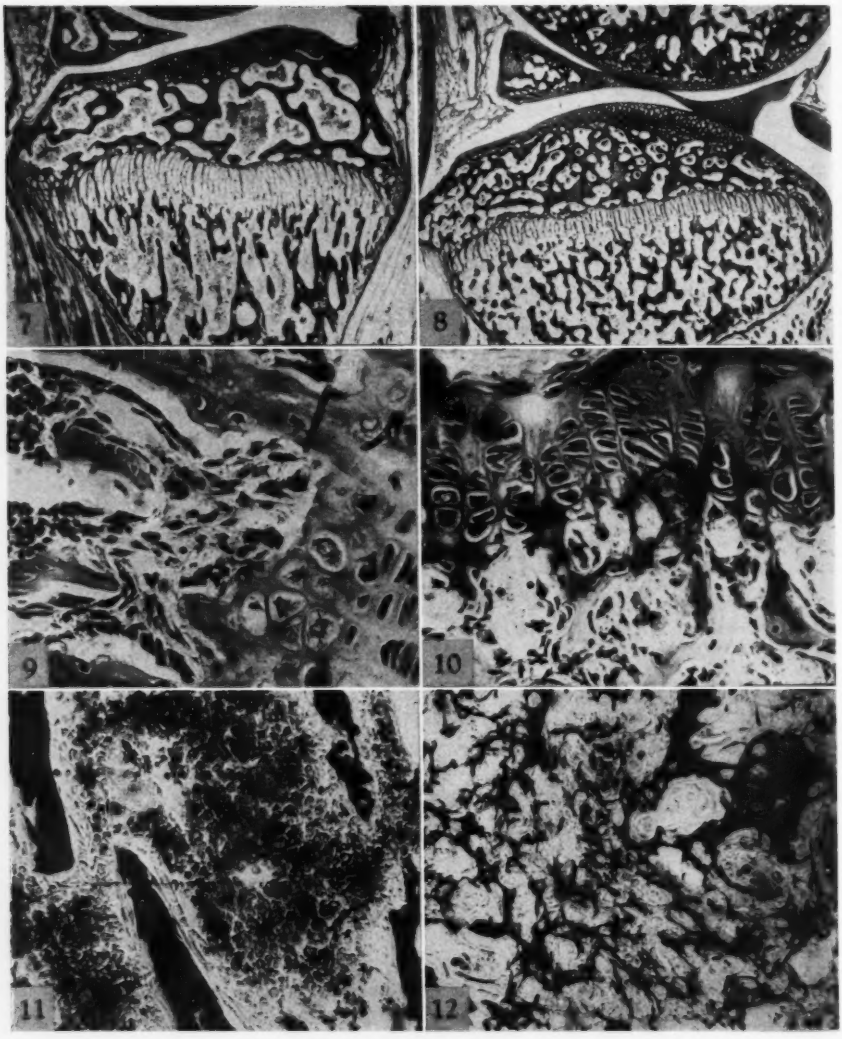
excluding most of the ilia, from an ovariectomized, estrogen-treated female. The cranio-dorsal and medial surfaces of the pubic bones have undergone marked resorption. Before fixation the interpubic gap measured 1.5 mm. Grossly, the patella and portions shown of the femur and tibia appear normal. (×3)

FIG. 5. A lateral portion of the pubic symphysis from an ovariectomized, untreated female. At the left is part of the symphysial fibrocartilage which is continuous with the articular cap of hyaline cartilage seen at the center. At the right is the medial end of the pubic bone consisting of trabeculae which surround small islands of hemopoietic tissue. Foot-Hortega silver stain. (×345)

FIG. 6. The tissue shown is from an ovariectomized, estrogen-treated female and is an area which corresponds to that shown in Fig. 5. The response of the symphysis was intermediate in this animal for the interpubic tissue contained both connective tissue and fibrocartilage. At the far left is part of some remaining fibrocartilage. Centrally, a network of reticular fibers is seen. This network occupies an area where the cartilagenous articular cap and pubic bone trabeculae existed before estrogen treatment. At the right is pubic bone. Its medial trabecular portion has been resorbed and endosteal bone growth has almost obliterated the medial-most portion of the marrow cavity. Foot-Hortega silver stain. (×345)



EXPLANATION OF FIGURES 7–12

FIG. 7. The proximal end of the tibia from an ovariectomized, untreated female. Foot-Hortega silver stain. (×40)

FIG. 8. The proximal end of the tibia and a portion of the distal end of the femur from an ovariectomized, estrogen-treated male. Extensive medullary bone formation occurred in the epiphysis and metaphysis at the expense of the marrow cavities. The epiphysial plate is thin and the bony spicules are irregularly arranged. Foot-Hortega silver stain. (×40)

FIG. 9. At the lower right is the epiphysial plate of the tibia from a castrated, untreated male. The marrow cavity of the metaphysis containing hemopoietic tissue and regularly-arranged bone spicules is at the left. (×345)

changes in both sexes. The structure and staining properties of the articular cartilage and ligaments of the knee resembled those of the controls. Some proliferation of connective tissue between the patellar ligament and the menisci was ascribed to a non-specific reaction to the estrone particles.

The estrogenic response at the knee varied. In some animals the patella, epiphyses and metaphyses revealed extensive medullary bone formation (Fig. 8). In others the patella and epiphyses showed little endosteal bone growth while the metaphyses showed much. The marrow cavities of the patella and epiphyses of the treated knee were almost completely replaced with coarse, irregularly-oriented spicules of bone in the animals showing an extensive effect. In the remaining marrow cavities only a layer of lining osteoblasts, capillaries and a few islands of hemopoietic tissue were present. The epiphysial plate in all the mice was similar in structure to the controls except that the layer of columnar cartilage cells averaged only 5 cells in thickness instead of 10. The range was 0–11 (Fig. 10). The metaphyses of the treated knee in each mouse were filled with coarse bone spicules. As in the epiphyses showing a marked effect, the surface of the spicules in the metaphyses was lined with a layer of osteoblasts and the normal marrow was largely replaced by a dense, vascular, connective tissue.

The Foot-Hortega method revealed many reticular fibers in the areas of the marrow cavities in the patella, femur and tibia where new bony spicules were probably being formed. The process was most pronounced in the metaphysis in both the femur and tibia (Fig. 12). The reticular fibers appeared to be continuous with the fibrous matrix of the spicules. They projected into the marrow from the ends of the spicules and were continuous with the network of reticular fibers of the remaining marrow. This marrow also contained local condensations of collagen among the reticular fibers which appeared to be independent of the spicules. The bony spicules in the control mice were arranged in paralled rows but in the estrogen-treated animals they were much coarser and showed no definite orientation with respect to the long axis of the bone. The marrow cavity of the diaphysis of both the tibia and femur of the treated knee showed a slight response in

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FIG. 10. At the upper half of the figure the thin epiphysial plate of the tibia from a castrated, estrogen-treated male is seen. At the lower half the metaphysis consists of irregularly-arranged bony spicules and marrow devoid of hemopoietic tissue. (×345)

FIG. 11. A portion of the metaphysis of the tibia from an ovariectomized, untreated female. Bone spicules and hemopoietic tissue are present but reticular fibers are absent. Foot-Hortega silver stain. (×345)

FIG. 12. A portion of the metaphysis of the tibia from a castrated, estrogen-treated male. At the upper right is a portion of a bone spicule. The hemopoietic tissue has been replaced by dense connective tissue, within which is a network of black-stained reticular fibers. Foot-Hortega silver stain. (×345)

some animals and none in others. The toluidine blue stain showed no significant differences in staining properties of the knees in this group when compared with those of the controls.

The estrogenic response was not limited to the local area of application since both humeri and the tissues of the contralateral knee showed the same morphological changes as were present in the knee containing the estrone implant.

DISCUSSION

Since estrone particles were still present at the implantation sites in the majority of mice at autopsy, the local concentration was assumed to be excessive. Estrogen concentration was adequate to systemically induce structural changes in the humeri and bones of the contralateral knee and the distension of the uterus. After local implantation of an estrone pellet in the distal portion of the marrow cavity of one femur, both femora responded (Halvorsen, 1949). However, the femurs which received the pellet showed the greatest response for the metaphysial and diaphysial marrow was almost completely replaced by bone. A difference in the response of the two femora was not observed in the present experiment, for the findings in both femora corresponded to the degree of response found in the contralateral knee of Halvorsen's study. This discrepancy in results might be accounted for by the fact that since Halvorsen made his implantations within the marrow cavity there was a more intense local effect than in the present study where the estrone was placed in contact with the periosteal surface. A strain difference in the mice and the difference in the amount of hormone used may also have contributed to the discrepancy.

The structural changes at the knee following local application of estrogen agreed with changes described after injecting the hormone subcutaneously in mice (Gardner and Pfeiffer, 1938; Sutro, 1940; Silberberg and Silberberg, 1941). The estrogenic response at the knee appeared to be limited to the formation of bony spicules within the marrow cavity, the replacement of hemopoietic tissue by dense connective tissue, and an inhibition of growth of the epiphysial cartilage as evidenced by its being thinner in the treated animals. The thinning of the epiphysial plate was previously reported following estrogen injections (Sutro, 1940; Silberberg and Silberberg, 1941; Gardner and Pfeiffer, 1943). Silberberg and Silberberg (1941) also reported a temporary hyperplasia and hypertrophy of the articular cartilage cells and a hyalinization and sclerosis of the cartilagenous ground substance following estrogen injection in mice of an age comparable to those used in the present experiment. Sutro (1940) reported that the cells of the articular growth plates at the knee in mice were retarded in their activity. In contrast, the ligaments, articular cartilages and menisci of the knee were virtually unchanged in the estrogen-treated mice of the present study when compared with the controls.

The pattern of pubic bone resorption in the present experiment was the same as that described following subcutaneous estrogen injections (Gardner, 1936; Hall, 1950; Crelin, 1954b). In contrast to the knee pronounced changes were noted in the interpubic tissue of the treated animals. In some mice the cartilage was completely replaced by an interpubic ligament. These findings are in agreement with those of Gardner (1936) and Crelin (1954c) but not with those of Hall (1950). She reported that after injecting 1.5 μg. of estrone daily from 8–20 days there was a resorption of pubic bone, but no formation of an interpubic ligament.

The area of the interpubic tissue adjacent to the pubic bones, where reticular fibers were found in the treated animals, was where Gardner (1936) described the proliferation of new connective tissue following estrogen treatment in mice. Even though this finding may be possible evidence for supporting the hypothesis that reticulum is a precursor of collagen, it cannot be regarded as definite since the observations were made only at the end of the 30-day period. The experiment now in progress, in which changes occurring daily during this period are being recorded, should yield more complete evidence.

The nature of the estrogenic response of the pubic symphysis in mice still remains unknown. The response is highly specific, for the pubic bones at the symphysis respond exactly opposite to other bones of the body. Also the connective tissue replacement of symphysial cartilage is unique since the ligaments and articular cartilages of the knee are not so affected, even with direct estrogen application. The unique manner of response appears to be a property of the symphysial tissue itself since it occurs identically by either local or systemic estrogen application. The presence of a cartilage and/or bone-resorbing enzyme secreted locally under estrogenic stimulation by an organ in the area of the symphysis to produce this specific response still cannot be excluded. The urinary bladder may influence the response for it is in direct contact with those portions of the symphysis which are affected first.

The results of the present experiment show that the gonads and their secretions need not be present for a sexual dimorphism of the bony pelvis to develop in mice.

The effect of estrogen on the urinary bladder was previously reported by Gardner (1935, 1936). In the present experiment the effect was most pronounced in the males. The urinary retention was probably a specific effect of estrogen rather than a result of injury to the urethra incidental to the operation, since none of the glass-treated mice showed any signs of retention or bladder hypertrophy.

SUMMARY

Five-week-old castrated male and female mice each received 2 implants of estrone particles: in contact with the dorsal surface of the pubic sym-

physis and under the patellar ligament of the left knee. Thirty days later the animals were sacrificed. Changes in estrogen-responsive tissues remote from the implants revealed that the latter were sufficient to produce a systemic effect. In each mouse the symphysis was flexible and the interpubic gap was measurable. Partial resorption of the pubes occurred in the same manner as that produced by subcutaneous estrogen injections. The degree of resorption of the pubic articular caps of hyaline cartilage and the replacement of the symphysial fibrocartilage by dense collagenous connective tissue was variable. In a number of each sex the interpubic tissue consisted almost entirely of ligamentous tissue directly connected to the pubic bones by networks of fine reticular fibers.

The cartilages and ligaments of the knee in each mouse were normal, however, medullary bone formation occurred in the patella and in the epiphysis and metaphysis of the femur and tibia. In these bone-forming regions the marrow cavities of a number of mice were almost completely replaced with coarse, irregularly-oriented bony spicules. An extensive proliferation of reticular fibers occurred in the areas of the marrow cavities where the bony spicules were probably being formed. The epiphysial plates were thinner than those of untreated control mice. The above changes also occurred in the untreated, contralateral knee.

Although 9-week-old, untreated, male and female control mice were castrated 3 weeks before puberty, a definite sexual dimorphism of their pelves developed.

REFERENCES

CRELIN, E. S.: *Proc. Soc. Exper. Biol. & Med.* **86**: 22. 1954a.

CRELIN, E. S.: *Am. J. Anat.* **95**: 47. 1954b.

CRELIN, E. S.: *Anat. Rec.* **120**: 1954c.

GARDNER, W. U.: *Proc. Soc. Exper. Biol. & Med.* **33**: 104. 1935.

GARDNER, W. U.: *Am. J. Anat.* **59**: 459. 1936.

GARDNER, W. U. AND C. A. PFEIFFER: *Proc. Soc. Exper. Biol. & Med.* **37**: 678. 1938.

GARDNER, W. U. AND C. A. PFEIFFER: *Physiol. Rev.* **23**: 139. 1943.

HALL, K.: *J. Endocrinol.* **5**: 174. 1947.

HALL, K.: *J. Endocrinol.* **7**: 54. 1950.

HALL, K.: *J. Endocrinol.* **7**: 299. 1951.

HALVORSEN, D. K.: *The Effect of Steroid Hormones on Bone.* Thesis submitted for M.D. degree. Yale U. Sch. of Med. 1949.

SILBERBERG, M. AND R. SILBERBERG: *Anat. Rec.* **80**: 347. 1941.

SUTRO, C. J.: *Proc. Soc. Exper. Biol. & Med.* **44**: 151. 1940.

REDUCTION OF PITUITARY CONTENT OF ACTH BY CORTISONE

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CORTISONE administration induces adrenocortical atrophy in experimental animals (Ingle and Kendall, 1937) and in man (Sprague *et al.*, 1950; Wilkins *et al.*, 1950). The mechanism of action of the steroid is in part a reduction in rate of discharge of ACTH from the adenohypophysis. However, the steroid has been reported to induce histopathological changes in the pituitary and the possibility exists that actual interference with ACTH synthesis as well as discharge occurs. The present report indicates that cortisone decreases the rate of synthesis of ACTH by the pituitary of the dog.

MATERIALS AND METHODS

Three mongrel dogs were treated with cortisone, 100 mg. per day, by subcutaneous injection. One animal was treated with cortisone for 14 days, a second for 44 days and a third for 71 days. Three dogs received injections of saline and served as controls. At the end of the treatment, each dog was anesthetized with sodium pentobarbital and killed by exsanguination. The pituitaries were removed as quickly as possible and the anterior lobes were frozen immediately upon removal. The frozen pituitary tissue was lyophilized, powdered and stored in a desiccator. The ACTH content of the glands was determined by the ascorbic acid-depletion method of Sayers *et al.* (1948). Male rats from the Sprague-Dawley farm, weighing 120 to 150 gm., were hypophysectomized 22 to 26 hours before assay. The left adrenal was removed and prepared for ascorbic acid analysis, and the solution to be assayed was injected intravenously in a volume of 1 ml. or less. One hour later the right adrenal was removed and prepared for ascorbic acid analysis. The difference in ascorbic acid concentrations between the left (control) and right (treated) adrenals was taken as a specific measure of ACTH activity. At the completion of the assay the sellae tursicae of the assay rats were exposed and the completeness of hypophysectomy determined by examination under 9× magnification. Results from incompletely hypophysectomized rats were discarded. Ascorbic acid was analyzed by the method of Mindlin and Butler (1937).

The pituitary tissue was dissolved in a few ml. of 0.9% sodium chloride solution, 0.01 N with hydrochloric acid, about 2 hours before injection and diluted with 0.9% sodium chloride solution to appropriate concentrations for assay. The assays were conducted on 3 assay days. A dose-response curve had been previously determined for lyophilized dog pituitary, which showed a linear regression of response with doses over the range 1.25

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to 20 μg. of lyophilized anterior pituitary. Accordingly, doses of 1.25, 5 and 10 μg. pituitary were employed on the first assay day. As will be seen from the results (Table 1) these doses were adequate for assay of normal pituitary, but were below the minimal dose necessary for a response with pituitaries from the 44-day and 71-day cortisone-treated dogs. Accordingly, the doses of pituitary from these dogs were increased to 20 and 80 μg. on the second day and finally to 80 and 160 μg. on the third assay day.

RESULTS AND DISCUSSION

The results are presented in Table 1. It is evident from these data that prolonged treatment with cortisone profoundly decreased the pituitary content of ACTH. There was no significant difference in the ACTH content of pituitaries from the 3 control dogs. Analysis of variance and estimations of relative potency on the assay data reveal that 1) the doses of lyophilized pituitary which were studied induced a response curve with a highly significant slope, 2) the slopes obtained from each of the pituitary preparations did not differ significantly from each other, 3) there was no significant departure from linearity in any instance. The 95% confidence limits for the potency estimates take into account both the assay error and the differences between control pituitaries, but reflect primarily the latter, which are believed to be the more valid error term for this estimate. The resulting confidence limits are consequently quite wide since there are only 2 degrees of freedom for estimating differences between controls. Even with the use of this maximum estimate of confidence limits, the pituitary from the dog which had received 100 mg. cortisone for 44 days was calculated to contain at most 9% the quantity of ACTH of the control pituitaries, while that from the dog which had been treated for 71 days contained at most 6% the amount of the controls.

TABLE 1. ASSAY[1] OF ACTH CONTENT OF ANTERIOR PITUITARY GLANDS FROM CORTISONE-TREATED AND CONTROL DOGS

Doses of lyophilized anterior pituitary, μg.	Cortisone-treated dogs (100 mg./day)			Control dogs (No cortisone)		
	14 day	44 day	71 day	1	2	3
1.25	44, 4, 34, 9	16, 12	−12, −3	64, 56, 33, 86	83, 37, 54, 72	62, 30, 81, 60
5.0	92, 110, 84, 89	17, −11, −16, 21	13, 6, −18, −16	103, 121, 114, 106	119, 83, 102, 146	91, 105, 95, 102
10.0	97, 94, 126, 122			122, 119, 152, 160	106, 119, 126, 125	111, 117, 102, 118
20.0		30, 59, 18, 29	33, 25, 9, −4			
80.0		85, 100, 68, 104	66, 102, 49, 34			
160.0		106, 102, 106, 84	108, 90, 96, 93			

[1] By the method of Sayers *et al.* Values given are depletion of adrenal ascorbic acid of the hypophysectomized rat, expressed as mg./100 gm. adrenal tissue.

Analysis of Variance

Source of variation	D.F.	S.S.	M.S.	F	P	Significant?
Among 3 control donors	2	1,109.55	554.78	1.06	.2−.1	no
Common regression, 6 donors	1	64,032.89	64,032.89	—	—	yes
Deviation from common regression	65	19,298.87				
Individual donor regression differences	5	1,799.80	359.96	1.20		no
Deviation of means from individual regressions	6	1,365.82	227.64	0.76		no
Within means (assay error)	54	16,133.25	298.76			

Estimate of potency, cortisone-treated/controls

Total dose of cortisone	Estimated potency	95% confidence limits of potency estimate
7.1 gm.	2.06%	0.78%– 5.46%
4.4 gm.	3.26%	1.23%– 8.65%
1.4 gm.	54%	20.3% –143%

Sayers *et al.* (1947) demonstrated that the injection of certain corticosteroids could decrease the *release* of ACTH. The above data is taken as evidence that the injected cortisone can markedly reduce *synthesis* of the trophic hormone. Had release of the hormone been suppressed, and synthesis continued at the same rate, accumulation of ACTH in the gland would have been expected. However, a decrease in ACTH content of the pituitary in the presence of a diminution of secretion is consistent only with a diminished rate of synthesis.

It is postulated that adrenocorticosteroids exert their pituitary depressing role in the homeostatic balance of ACTH release and adrenocortical response by depressing not only the release of ACTH but the synthesis of this trophic hormone as well.

The observed profound decrease in pituitary ACTH content following cortisone treatment adds further evidence that the adrenocortical atrophy reported in long-term cortisone therapy (Sprague *et al.*, 1950; Wilkins *et al.*, 1950) is mediated by suppression of pituitary activity.

SUMMARY

Dogs were treated with cortisone, 100 mg. per day, for 14, 44 and 71 days. The pituitaries were removed and assayed for ACTH content as compared to controls. The ACTH content was found to be depressed to 54% of control levels in 14 days, 3% in 44 days and 2% in 71 days.

Acknowledgment

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REFERENCES

Ingle, D. J. and E. C. Kendall: *Science* **86:** 245. 1937.

Mindlin, R. L. and A. W. Butler: *J. Biol. Chem.* **122:** 673. 1937.

Sayers, M. A., G. Sayers and L. A. Woodbury: *Endocrinology* **42:** 379. 1948.

Sayers, G. and M. A. Sayers: *Endocrinology* **40:** 265. 1947.

Sprague, R. G., Power, M. H., Mason, H. L., Albert, A., Mathieson, D. R., Hench, P. S., Kendall, E. C., Slocumb, C. H. and H. F. Polley: *Arch. Int. Med.* **85:** 199. 1950.

Wilkins, L., Crigler, J. F., Jr., Silverman, S. H., Gardner, L. I. and C. J. Migeon: *J. Clin. Endocrinol.* **11:** 1. 1951.

ELECTROPHORETIC STUDIES ON THE DISTRIBUTION OF HEXOSAMINE IN PROTEINS OF THE THYROID GLAND

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THE existence of glycoproteins in the thyroid gland has been suggested on the basis of histochemical studies (Fisher, 1953; Glegg, Clermont, and Leblond, 1952; Gersh, 1950; Levine, 1950). With the exception of isolated glucosamine and hexose determinations on thyroglobulin (Bland Kassell and Heidelberger, 1939; Glegg, Clermont and Leblond, 1952) and the qualitative identification of mannose and galactose in whole thyroid tissue (Werner, 1953), few direct chemical studies have been applied to the distribution of sugars in the thyroid gland.

In the course of a study on the distribution of hexosamines[2] in body tissues, it was noted that thyroid glands contained relatively large concentrations of hexosamine (Boas, 1953). In order to interpret this and to identify the hexosamine-rich substance or substances in the thyroid, electrophoretic studies were carried out on extracts of hog thyroid. The distribution of hexoses and uronic acid were also studied in an attempt to characterize further the glycoproteins present. Animal studies were carried out to study the influence of hypophysectomy, thyrotropic hormone and stress on the amount of thyroid hexosamine.

METHODS

Fresh hog thyroids were delivered in a frozen state from a local slaughter house. They were immediately placed in flasks and lyopholyzed until free of water. The dry fatty chunks were ground in a Waring Blendor prior to extraction.

pH 8.6 Extraction.—2.75 gms. of ground thyroid were extracted for 24 hours at 4° C. with 40 ml. of pH 8.6 veronal buffer (ionic strength 0.06). Following centrifugation, the residue was re-extracted and separated four times in a similar fashion. The final residue was desalted, defatted and dehydrated by washing with progressively increasing concentrations of acetone (70–100%). The final weight was 329 mg. The fat removed represented 51% of the starting material. The veronal extracts were pooled, dialyzed against water and lyopholized, yielding 1010 mg. of powder.

pH 4.5 Extraction.—2.75 gm. of ground thyroid were extracted with pH 4.5 sodium

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[2] The two naturally occurring hexomsamines are glucosamine and galactosamine. The method used here does not distinguish between these amino sugars.

acetate-acetic acid, sodium chloride buffer (ionic strength 0.1) (Mehl, 1950) according to the scheme used for veronal. The extract powder weighed 76 mg. and the defatted residue 1294 mg. The fat removed represented 50% of the starting material.

Electrophoresis.—Paper electrophoresis was carried out on a horizontal apparatus (Arthur Thomas Co.) using Whatman No. 1 filter paper sheets. A 10% solution of an unknown sample was applied evenly on a 150 mm. starting line in a volume of 0.10 ml. Each separation was run for 5–6 hours at 8–10 mA and 250–200V in pH 8.6 veronal buffer, or at 10 mA and 20V in pH 4.5 acetate-sodium chloride buffer.

Protein.—To determine the distribution of proteins following electrophoresis the paper was dried in an oven at 110° C. for twenty minutes. A narrow longitudinal strip was cut out and stained for seven minutes in an alcoholic solution of 0.1% bromphenol blue saturated with mercuric chloride (Durrum, 1950), rinsed in tap water for five minutes and dried in an oven at 110° C. The strip was then read at 600 mμ on a Beckman spectrophotometer (Model DU) by means of a specially constructed strip holder. A protein distribution curve was obtained by charting optical density (ordinate) against distance migrated (abscissa) on graph paper. The protein distribution was estimated by cutting out the charted electrophoretic curve, weighing it and relating the weight of each component to the total weight.[3]

Nitrogen.—The nitrogen content of samples was determined by a Kjeldahl micromethod.

Hexosamine.—The electrophoretic distribution of hexosamine was determined by cutting off bands of unstained but oven-dried paper corresponding to the protein peaks determined on parallel strips. Each band of paper was hydrolyzed in 1N hydrochloric acid for 15 hours and after isolation with Dowex-50, hexosamine was determined by a modification of the method of Elson and Morgan (Boas, 1953). The residues, extracts, and thyroids from other animals were hydrolyzed in 3N hydrochloric acid for fifteen hours and treated similarly.

Hexoses.—Hexoses were determined by a modification of the anthrone method (Kapuscinski and Zak, 1953) using an equimolar glucose-mannose-galactose standard. In order to determine the electrophoretic distribution of hexoses, the paper was dried by lyopholization and using pH 8.6 veronal buffer the proteins were eluted from bands corresponding to the protein peaks determined on parallel strips. After filtering, these eluates were used in the anthrone method.

Uronic Acid.—Uronic acid was determined on samples of the residues and extracts by a modification of the carbazole method of Dische (1946) which utilized a tissue blank in correcting for most of the non-specific color development and in which the standard of reference was the difference between the readings of two duplicate tissue samples, with and without glucuronolactone. All of the uronic acid values reported are higher than their actual content, since it was necessary to use relatively large amounts of these samples to detect the small amounts of uronic acid present. This resulted in the development of some non-specific background absorption for which a correction could not be made.

Iodine.—Iodine determinations were performed by the chloric acid method (Zak, *et al.* 1952) on protein samples and on unstained paper following electrophoretic separation of the proteins.

Animal Experiments.—Male, Sprague-Dawley rats were individually caged, fed a stock diet (Boas and Peterman, 1953) and permitted to drink water *ad libitum.* Hypophysectomy was performed by the parapharyngeal approach under ether anesthesia. All hypophysectomized rats and their controls received injections of 5000 units of penicillin a day. Two pituitary hormone preparations containing thyrotropic hormone

[3] No corrections have been made for the variation in dye-binding capacity of the different proteins.

activity,[4] were injected subcutaneously daily. The animals were killed with ether and completeness of hypophysectomy determined by examination of the sella turcica under magnification. Only completely hypophysectomized rats are included here. In one experiment in which the effect of stress was studied the right femur and tibia were fractured under light ether anesthesia.

RESULTS

Hexosamine in Thyroids.—Normal thyroid glands from several species of animals and a sample of human non-toxic nodular goiter were analyzed for their hexosamine content (Table 1). The rat and chick had the lowest

TABLE 1. HEXOSAMINE CONTENT OF THYROIDS OF SEVERAL SPECIES

Animal	Hexosamine (% dry wt.)*
Rat	1.04
Chick	1.28
Human	1.38
Dog	1.48
Hog	1.83
Monkey	1.95
Guinea Pig	2.10

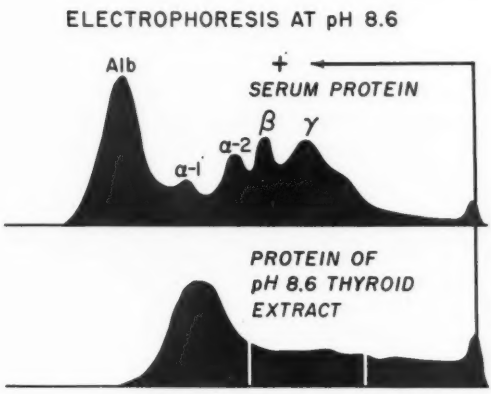
* Acetone dried tissue.

concentrations and the hog, monkey and guinea pig had the highest. All, however, had appreciable amounts of hexosamine varying from 1–2% of the dry weight.

pH 8.6 Extract.—In order to extract thyroglobulin, pH 8.6 veronal buffer was used. The veronal-soluble extract represented 75.8% of the total dry fat-free thyroid (Table 2). In addition, this fraction contained 92.6% of the total thyroid hexosamine, 87.9% of the total thyroid hexose but essentially no uronic acid (3.2%). This fraction also contained 98.3% of the total iodine, demonstrating almost complete extraction of thyroglobulin. The residue contained most of the uronic acid (96.8%). but this represented a concentration of less than 0.19%.

Electrophoresis of pH 8.6 Extract.—To study the composition of this extract, replicate samples were separated electrophoretically and analyzed for hexosamine, hexose and iodine (Fig. 1). A large protein peak developed which represented 49.0% of the protein in the extract and with a mobility similar to alpha-globulin of serum. On analysis this was found to contain 78.0% of the total hexosamine and 69.8% of the total hexose in the extract. This peak was identified as thyroglobulin since it contained over 95.0% of the total iodine in the extract and had a mobility identical to a

[4] Thyrotropic hormone—Armour Lot No. 129-31PRR3, kindly supplied by Dr. Irby Bunding, Armour Laboratories, Chicago. Growth hormone, No. C_5 I_5, E. R. Squibb and Sons. Contains thyrotropic activity in the dosage administered. Supplied by Dr. Robert W. Bates.



	DISTRIBUTION (% OF EXTRACT)		
PROTEIN	49.0	26.1	24.9
HEXOSAMINE	78.0	12.7	9.3
HEXOSE	69.8	5.7	24.5
IODINE	>95.0	TRACE	TRACE

FIG. 1. Electrophoresis of pH 8.6 extract of hog thyroid and normal human serum in pH 8.6 veronal buffer.

known thyrogobulin preparation (CH-29).[5] The slower moving components probably represented nucleoproteins which are present in thyroid gland extracts (Heidelberger and Palmer, 1933). When the pH 8.6 extract was subjected to electrophoresis in pH 4.5 acetate-NaCl buffer, it remained almost entirely immobile on the starting line. A small slow-moving pro-

TABLE 2. CHEMICAL ANALYSIS OF pH 4.5 (ACETATE-NaCl BUFFER) AND pH 8.6 (VERONAL BUFFER) EXTRACTS OF HOG THYROID

Extracting agent	Fraction	Per cent distribution				
		Dry wt.	Hexos-amine	Iodine	Uronic acid	Hexose
pH 8.6 Veronal buffer	Extract	75.8	92.6	98.3	3.2	87.9
	Residue	24.2	7.4	1.7	96.8	12.1
pH 4.5 NaCl-Acetate buffer	Extract	5.4	16.7	0.8	16.0	10.5
	Residue	94.6	83.3	99.2	84.0	89.5
		Concentration (% dry wt.)				
pH 8.6 Veronal buffer	Extract	—	2.10	0.72	<0.02	3.14
	Residue	—	0.51	0.04	<0.19	1.33
ph 4.5 NaCl-Acetate buffer	Extract	—	5.88	0.10	<0.28	6.80
	Residue	—	1.72	0.61	<0.08	3.36

[5] Hog thyroglobulin, CH-29, Lot No. 91921 generously supplied by Dr. Robert L. Kroc of Warner-Chilcott Laboratories, Morris Plains, N. J.

tein band migrating toward the anode, however, almost invariably appeared. It stained faintly with bromphenol blue and contained hexosamine.

Analysis of Thyroglobulin.—According to the analytical data obtained by electrophoresis, the thyroglobulin contained 3.3% hexosamine, 4.5% hexose and insignificant amounts of uronic acid (Table 3). When expressed in terms of the distribution of these substances in whole thyroid, thyroglobulin contained 37.0% of the total thyroid protein, 72.2% of total thyroid hexosamine, 61.4% of total thyroid hexoses, insignificant amounts of uronic acid and essentially all of the iodine.

pH 4.5 Extract.—Since blood thyroxin is carried by the carbohydrate-rich serum alpha-globulins (Gordon, Gross, O'Connor, and Pitt-Rivers, 1952) and since the thyroxin-binding protein (Robbins, Petermann and

TABLE 3. ANALYSIS OF ELECTROPHORETICALLY-SEPARATED THYROGLOBULIN

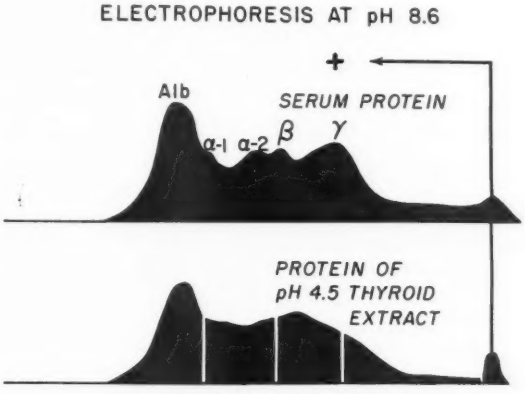
	Thyroglobulin (% dry wt.)	Distribution (%)*	
		Thyro-globulin	Rest of thyroid
Protein (as N×6.25)	85.5†	37.0	63.0
Hexosamine	3.34	72.2	27.8
Hexose	4.50	61.4	38.6
Uronic Acid	<0.02	3.2	96.8
Iodine	1.42	>93.5	< 6.5

* Calculations based on data in Table 2 and Figure 1.
† Based on analysis of pH 8.6 veronal extract. May be slightly higher for thyroglobulin since slower moving components are probably nucleoproteins.

Rall) and alpha and beta globulin mucoproteins can be electrophoretically separated from the other serum proteins at pH 4.5 (Mehl, 1950), the question of the presence of similar acid-soluble mucoproteins in the thyroid was examined. Hog thyroid was extracted with pH 4.5 acetate-sodium chloride buffer. Thyroglobulin has an isoelectric point of pH 4.5 (Heidelberger and Pedersen, 1935–6), so would be almost completely insoluble under these conditions. The soluble pH 4.5 extract contained only 5.4% of the weight of dry defatted thyroid, so represented only a small fraction of the thyroid (Table 2). Since the extract contained only 0.8% of the total thyroid iodine, thyroglobulin remained almost entirely in the residue. The amount of the total thyroid hexosamine and hexose in this fraction was also small, which was expected since most of the hexose and hexosamine was in the thyroglobulin fraction. The pH 4.5 extract had a high concentration, however, of hexosamine (5.88%) and hexose (6.8%) but had only small amounts of uronic acid (less than 0.27%).

Electrophoresis of pH 4.5 Extract.—When the pH 4.5 extract was separated electrophoretically in pH 8.6 veronal buffer, the protein migrated as two major peaks (Fig. 2). One had a mobility similar to albumin and the other that of a slower moving globulin. Since the faster moving com-

ponent was not present in the pH 8.6 veronal extract run under the same conditions, it probably was not albumin. The slower moving proteins did not appear to represent thyroglobulin unless it were in a denatured or altered form, since thyroglobulin is insoluble under these conditions and since this fraction contained only 0.1% iodine. Hexosamine was distributed over the entire fraction but with higher concentrations (7.6–8.8%) in the



	DISTRIBUTION (% OF EXTRACT)				
PROTEIN	30.4	21.7	26.8	18.9	2.2
HEXOSAMINE	21.6	32.3	34.8	10.4	0.9
	CONCENTRATION (% PROTEIN)				
HEXOSAMINE	4.2	8.8	7.6	3.2	2.5

FIG. 2. Electrophoresis of pH 4.5 extract of hog thyroid and normal human serum in pH 8.6 veronal buffer.

proteins with mobilities similar to the serum globulins. This extract, therefore, was very rich in hexosamine and hexose, and may have represented either a chemically altered thyroglobulin or an unidentified glycoprotein. The extract did not appear to contain either hyaluronic acid or chrondroitin sulfate in any appreciable amounts since the concentration of uronic acid in the extract was very low (0.28%) (Table 2).

In an effort to characterize further this glycoprotein the pH 4.5 extract was separated electrophoretically in pH 4.5 acetate-sodium chloride buffer (Figure 3). Three protein peaks of low mobility appeared. The fastest moving component had a higher hexosamine concentration (11.5%) than any thyroid protein examined. This glycoprotein appeared to be distinct from thyroglobulin and, as suggested when separated in pH 8.6 buffer, it may have represented a chemically altered thyroglobulin, or unidentified glycoprotein with some similarities to the mucoprotein fractions of serum (Mehl, 1950) and the thyroxin-binding protein of serum (Robbins, Peter-

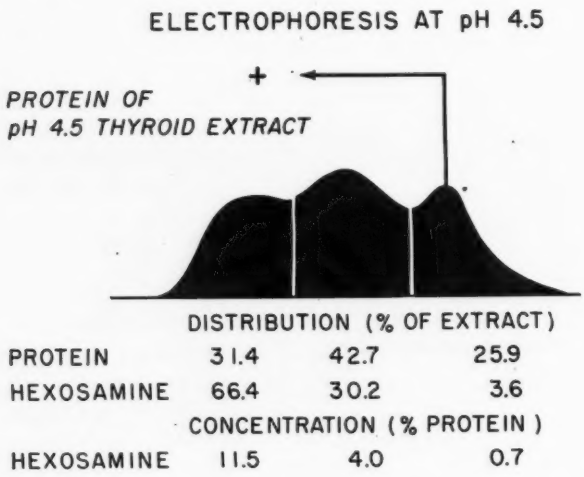


FIG. 3. Electrophoresis of pH 4.5 extract of hog thyroid in pH 4.5 acetate-NaCl buffer.

mann and Rall). In amount, this hexosamine-rich, fast-moving component in pH 4.5 buffer represented only 1.7% of the total thyroid protein.

Hypophysectomy.—Since it was shown that thyroid hexosamine was largely contained in thyroglobulin, it became of interest to study the effects of physiological situations known to affect profoundly the functional state of the thyroid. In Experiment I, rat thyroids studied seven days following hypophysectomy were significantly smaller than those in unoperated controls (Table 4). Their total hexosamine content (μg.) was unchanged. As a consequence of the weight changes, the concentration of hexosamine increased significantly.

Thyrotropic Hormone (TSH).—In Experiment II two preparations of pituitary hormones known to contain TSH activity were given to hypo-

TABLE 4. EFFECT OF HYPOPHYSECTOMY AND TREATMENT WITH PITUITARY HORMONES ON THYROID HEXOSAMINE*

Expt.	Group	No. rats	Body wt. (gm.)	Thyroid (mg.)	Hexosamine	
					Total (μg.)	Mg./100 gm.
I	Control	5	138 ± 7	9.5 ± 0.7	24.6 ± 1.2	257 ± 14
	Hypophysectomy	6	99 ± 4	5.7 ± 0.2	21.1 ± 0.8	369 ± 13
II	Hypophysectomy	4	100 ± 3	6.2 ± 0.1	25.5 ± 1.6	410 ± 23
	Hypophysectomy—TSH†	4	104 ± 8	11.1 ± 1.0	26.5 ± 2.1	244 ± 26
	Hypophysectomy—GH‡	3	135 ± 2	10.9 ± 1.2	28.3 ± 2.5	260 ± 7

* Mean ± S.E. All rats treated 7 days from day of operation.
† TSH given 3 mg. per day in corn oil with aluminum monostearate.
‡ GH given 2 mg. per day in water.

physectomized rats for seven days from the day of operation (Table 4). Both treated groups developed significant increases in thyroid weight but no change in the total thyroid hexosamine. As a consequence, the concentration of thyroid hexosamine decreased significantly.

Stress (fracture).—To test the influence of stress on thyroid function, rats were killed at intervals (1, 2, 4 and 9 days) following experimental fractures and the thyroid glands examined. No significant changes in total hexosamine or the concentration of hexosamine resulted. The thyroid weight changes were attributable to the growth of the animals during this period (Table 5).

DISCUSSION

Thyroid glands from several species of animals have uniformly demonstrated high concentrations of hexosamine. It has been shown that in hog thyroid 72% of the total thyroid hexosamine is present in thyroglobulin.

TABLE 5. EFFECT OF STRESS (FRACTURE) ON THYROID HEXOSAMINE*

Days after fracture	No. rats	Body wt. (gm.)	Thyroid (mg.)	Thyroid	Hexosamine	
				mg./100 gm. Body wt.	Total (μg.)	Mg./100 gm.
0	7	105 ± 3	9.5 ± 0.6	9.0 ± 0.3	27.1 ± 1.1	287 ± 9
1	7	113 ± 4	9.8 ± 0.3	8.7 ± 0.6	27.2 ± 1.9	279 ± 18
2	7	107 ± 3	8.8 ± 0.3	8.2 ± 0.3	23.6 ± 1.8	270 ± 20
4	7	120 ± 4	11.0 ± 0.6	9.2 ± 0.2	29.3 ± 2.1	266 ± 13
9	6	137 ± 5	12.0 ± 0.2	8.8 ± 0.2	30.8 ± 1.7	257 ± 18

* Mean ± Standard Error.

Total thyroid hexosamine may therefore provide a reasonable index of the amount of thyroglobulin present. The value of 3.3% hexosamine reported here in thyroglobulin is slightly higher than the values reported by Brand, Kassell, and Heidelberger (1939) and by Glegg, Clermont, and Leblond (1952). In addition, it was found that hog thyroglobulin contained 4.5% hexose. Although identified, no figures were given for hexoses by Brand, Kassell, and Heidelberger (1939). Since thyroglobulin is electrophoretically homogeneous (Kamal and Turner, 1951; Derrien, *et al.*, 1949) it appears that thyroglobulin is a glycoprotein, a finding in agreement with the histochemical observations of Gersh (1950) and Glegg, Clermont, and Leblond (1952).

A small acid-soluble mucoprotein fraction has been demonstrated in the thyroid. It is very rich in hexosamine (11.5%) and its electrophoretic mobility in pH 4.5 buffer is similar to the mucoprotein fraction (MP-2) of Mehl (1950) and thyroxin-binding protein (Robbins, Petermann and Rall). The pH 4.5 extract which contained this mucoprotein also contained hexoses and iodine (0.1%).

On the basis of the analytical data presented here there is little evidence

to support the histochemical observations that the thyroid gland contains hyaluronic acid (Gersh, 1950, Levine, 1950) or other mucopolysaccharides (Fisher, 1953). All of the fractions studied contained only barely detectable quantities of uronic acid. In whole dry hog thyroid the uronic acid concentration was less than 0.08%, giving a hexosamine—uronic acid ratio of greater than 23:1. If the uronic acid represented mucopolysaccharides of the thyroid cells or colloid it is unlikely that there would be sufficient to given any histochemical staining reaction. The small amounts of uronic acid present could more readily be explained on the basis of a mucopolysaccharide of the thyroid connective tissue which would also not normally stain.

If total thyroid hexosamine is a reliable index of changes in total thyroid thyroglobulin, it would appear that thyroglobulin cannot easily be altered in amounts by either hypophysectomy or the administration of TSH. Although it is well known that the iodine content of the thyroid decreases following stimulation with TSH, this does not necessarily reflect a decrease in thyroglobulin since the iodine content of thyroglobulin varies with different physiological states (Derrien, Michel, and Roche, 1948).

SUMMARY

Thyroid glands of various species of animals and man have a high concentration of hexosamine (1–2% dry wt.). Thyroglobulin is shown to contain most of the hexosamine (72%) as well as thyroid hexoses (61%) but no uronic acid. Thyroglobulin is therefore a glycoprotein, rich in hexosamine (3.3%) and hexose (4.5%).

An acid-soluble glycoprotein has also been demonstrated in the thyroid gland. It is present in small amounts and is very rich in hexosamine (11.5%).

The evidence presented does not support the observations that the thyroid gland (exclusive of connective tissue) contains a mucopolysaccharide.

Hypophysectomized rats or rats treated with thyrotropic hormone, despite changes in thyroid weight, develop no changes in total thyroid hexosamine.

Stress (fracture) does not influence the total amount or concentration of hexosamine in the thyroid.

Acknowlegments

We wish to thank Dr. Robert W. Bates of the National Institutes of Health for performing the iodine determinations, Dr. William Newman of the George Washington University Hospital, D. C., for supplying the human thyroid specimen and Miss Mary P. Brosnan for carrying out the hexose determinations.

REFERENCES

BOAS, N. F.: *J. Biol. Chem.* **204**: 553. 1953.

BOAS, N. F. AND A. F. PETERMAN: *Proc. Soc. Exp. Biol. Med.* **82**: 19. 1953.

BRAND, E., B. KASSELL AND M. HEIDELBERGER: *J. Biol. Chem.* **128:** xi. 1939.
DERRIEN, Y., R. MICHEL, K. O. PEDERSEN AND J. ROCHE: *Bioch. and Bioph. Acta* **3:** 436. 1949.
DERRIEN, Y., R. MICHEL AND J. ROCHE: *Bioch. and Bioph. Acta* **2:** 454. 1948.
DISCHE, Z.: *J. Biol. Chem.* **167:** 189. 1946.
DURRUM, E. L.: *J. Am. Chem. Soc.* **72:** 2943. 1950.
FISHER, E. R.: *Arch. Path.* **56:** 275. 1953.
GERSH, I.: *J. Endocrin.* **6:** 282. 1950.
GLEGG, R. E., Y. CLERMONT AND C. P. LEBLOND: *Stain Tech.* **27:** 277. 1952.
GORDON, A. H., J. GROSS, D. O'CONNOR AND R. PITT-RIVERS: *Nature* **169:** 19. 1952.
HEIDELBERGER, M. AND W. W. PALMER: *J. Biol. Chem.* **101:** 433. 1933.
HEIDELBERGER, M. AND K. O. PEDERSEN: *J. Gen. Physiol.* **19:** 95. 1935–6.
KAMAL, S. AND C. W. TURNER: *J. Dairy Sc.* **34:** 798. 1951.
KAPUSCINSKI, V. AND B. ZAK: *Am. J. Clin. Path.* **23:** 784. 1953.
LEVINE, M. D.: *J. Endocrin.* **6:** 288. 1950.
MEHL, J. W. AND F. GOLDEN: *J. Clin. Invest.* **29:** 1214. 1950.
ROBBINS, J., PETERMANN, M. L. AND RALL, J. E.: *J. Biol. Chem.* In press.
WERNER, I.: *Acta Soc. Med. Upsal.* **58:** 1. 1953.
ZAK, B., H. WILLARD, G. B. MYERS AND A. BOYLE: *J. Anal. Chem.* **24:** 1345. 1952.

THE DESTRUCTION OF INSULIN BY INTACT MICE[1]

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IT HAS been demonstrated that insulin is destroyed "in vitro" by slices, homogenates and extracts of liver (Mirsky and Broh-Kahn, 1949; Mirsky and Perisutti, 1953; Mirsky, Perisutti and Dixon, 1954; Mirsky, Perisutti and Dixon, 1955; Tomizawa, Nutley, Marahara and Williams, 1954). Although it is generally agreed that insulin is destroyed also by the intact animal (Greely, 1940; Mirsky, Podore, Wachman and Broh-Kahn, 1948; Haugaard, Vaughan, Haugaard and Stadie, 1954), the evidence of such destruction is quite indirect. In order to obtain more definitive evidence of insulin destruction "in vivo," the rate of degradation of I^{131} labeled insulin by the intact mouse was determined.

METHOD

Fed male mice of from 18 to 25 gm. in weight were given an intraperitoneal injection of two units insulin per 100 gm. of body weight and a subcutaneous injection of 1 ml. of a 5.5% glucose solution. The total volume of insulin injected intraperitoneally was 0.02 ml. per gram body weight. The insulin consisted of a mixture of crystalline zinc insulin and tracer quantities of I^{131} labeled insulin prepared by the Abbott Laboratories. One group of mice was sacrificed immediately after the injection. Other animals were sacrificed at 30, 60 and 120 minutes after the injection of insulin during which intervals they were kept in individual beakers. Each group consisted of ten mice. Those mice which were sacrificed at 120 minutes after the injection of insulin were given an additional injection of 1 ml. glucose at 60 minutes. At the end of the designated interval, each mouse was cut up with shears into a Waring Blendor and homogenized with three volumes 10% trichloracetic acid. The contents of each animal's beaker were washed into the Blendor with the trichloracetic acid. The total mixture was divided up into approximately 20 ml. aliquots in tared 50 ml. plastic centrifuge tubes and the weight of each aliquot was determined. The radioactivity of the aliquots was then measured in a well-type gamma counter. Immediately thereafter the aliquots were filtered into tared tubes, the filtrates weighed and the radioactivity of each filtrate measured. All counts were corrected for background and coincidence and the counts per minute per gram computed. The percentage of counts in the trichloracetic acid filtrate of each aliquot of homogenate was computed.[2] The mean percentage of the I^{131} released into the non-protein fraction

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[2] Subsequent studies revealed that identical results are obtained with the following procedure: Two 20 ml. aliquots of the homogenate are weighed. The remainder of the homogenate is filtered and two 20 ml. aliquots of the filtrate weighed. The radioactivity of the aliquots is determined and expressed as counts per minute per gram.

was used to compute the fraction of the administered insulin which was destroyed by each mouse in the designated interval. Appropriate corrections for the destruction of insulin which occurred during the homogenization of the mouse were made by subtracting the value obtained in mice sacrificed immediately after the injection from the values obtained at 30, 60 and 120 minutes after injection.

In addition to the above, groups of three to six mice were injected intraperitoneally with different amounts of insulin containing the same amount of I^{131} labeled insulin and subcutaneously with 1 ml. of 5.5% glucose. One group of animals was sacrificed immediately and another group in 30 minutes after the injections. After subtracting the percentage of non-protein I^{131} found in the animals sacrificed immediately after injection, the quantity of insulin destroyed by each animal in the interval was computed from the percentage of I^{131} released into the non-protein fraction.

RESULTS

In order to determine whether different aliquots of homogenates of mice are sufficiently constant to permit the calculation of the quantity of insulin destroyed by the intact animal, the homogenates of several mice which were previously injected with labeled insulin were pooled and divided into seven aliquots. The radioactivity of each aliquot and of the non-protein fraction was measured as described above. The data thus obtained indicate that aliquots of a single homogenate are quite constant (Table 1), the coefficient of variablity being 3.8%.

The data illustrated in Figure 1 represent the mean percentage of I^{131} present in the non-protein fraction of homogenates of whole mice after correcting for the I^{131} present in the non-protein fraction of animals sacrificed immediately after the injections of insulin and glucose. It is apparent that a significant progressive increase in the percentage of non-protein I^{131} occurs with time. Since this increase represents the I^{131} that was released from the injected insulin and parallels the rate of insulin inactivation (Mirsky, Perisutti and Dixon, 1954) it serves as a measure of the quantity of insulin degraded during the designated interval. Accordingly, the mean ($\pm$S. E.) rate of destruction was $14.5 \pm 0.62\%$ in 30 minutes, $33.3 \pm 1.88\%$ in 60 minutes and $62.0 \pm 2.30\%$ in 2 hours. Since each mouse was given 2

TABLE 1. VARIABILITY BETWEEN ALIQUOTS OF TRICHLORACETIC ACID HOMOGENATES OF MICE

Aliquots of homogenate		Filtrate of aliquots		Per cent of total in filtrate
Grams	Counts/min./gm.	Grams	Counts/min./gm.	
15.4670	1480	8.6089	265	17.9
16.3457	1525	7.9902	279	18.3
18.1844	1481	9.3331	268	18.1
12.8774	1513	5.7892	257	17.0
11.3650	1518	5.4260	261	17.2
16.7730	1521	8.9136	260	17.1
18.7755	1481	9.1982	277	18.7
Mean $\pm$S.D.				17.8 ± 0.67

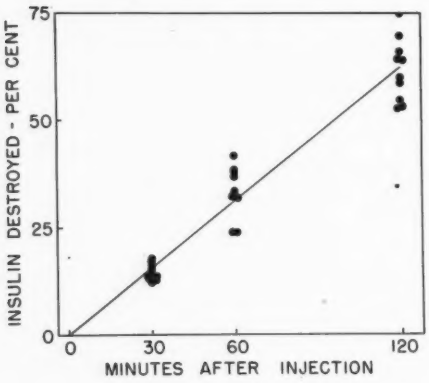

FIG. 1. The Rate of Degradation of I^{131} Labeled Insulin by Intact Mice. Per cent of total I^{131} found in non-protein fraction of homogenates of mice at different intervals after the injection of I^{131} labeled insulin. All values corrected for that found immediately after injection. The mean rate computed from the regression of the per cent of non-protein I^{131} on time ($Y = 0.18 \pm 0.52X$).

units of insulin per 100 gm., the rate of insulin destruction was 0.28, 0.66 and 1.24 units per 100 gm. in 30, 60 and 120 minutes respectively.

In order to determine the relationship between the rate of insulin degradation and the quantity of insulin administered, increasing amounts of insulin were administered to groups of three to six mice and the former computed from the percentage of I^{131} released into the non-protein fraction in 30 minutes. It is evident from Figure 2 that up to a concentration of from 30 to 40 units per 100 gm., the quantity of insulin destroyed in 30 minutes after the injection is related directly to the quantity of insulin injected. A Lineweaver-Burk calculation (Lineweaver and Burk, 1934) of these data suggests that the degradation of insulin "in vitro" follows first order kinetics (Insert Figure 2). The maximum velocity, Vm, thus derived is 59.35 units per 1000 gm. of mouse per 30 minutes and the Michaelis constant, Km, is 409.03. Assuming a molecular weight of 6000 and expressing the initial velocities as the mM of insulin destroyed per 1000 gm. of mouse in 30 minutes and the concentration of insulin injected as mM per 1000 gm., the Vm and Km thus derived are 3.87×10^{-4} and 2.73×10^{-3} respectively.

DISCUSSION

The destruction of insulin by slices, homogenates and extracts of liver has been attributed to the action of an apparently specific hydrolytic enzyme system which has been tentatively designated as "insulinase" (Mirsky and Broh-Kahn, 1949; Mirsky, Perisutti and Dixon, 1955).

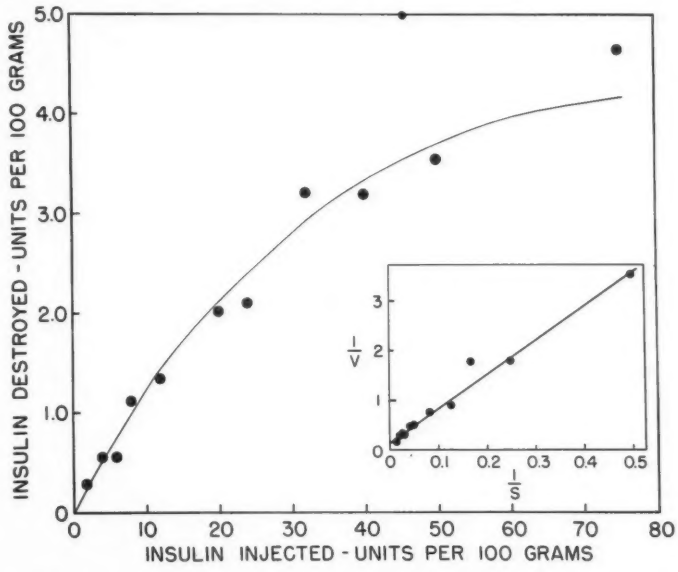

FIG. 2. Effect of the Quantity Injected on the Degradation of I^{131} Labeled Insulin by the Intact Mouse. The quantity of insulin destroyed in 30 minutes computed from the per cent of total I^{131} found in non-protein fraction of homogenates of mice. All values corrected for that found immediately after injection. Insert is a Lineweaver-Burk plot of the data in which V = units I^{131} labeled insulin degraded in 30 minutes per 100 grams of mouse and S = units I^{131} labeled insulin administered per 100 grams of mouse.

This system follows first order kinetics with a Km that is characteristic of hydrolytic systems. The data reported herein suggest that the degradation of insulin in the intact mouse is relatively rapid and follows first order kinetics. Accordingly, it is probable that the system responsible for the degradation of insulin "*in vitro*" is also responsible for the degradation of insulin "*in vivo*."

The rapidity with which exogenous insulin is destroyed by the intact animal and the quantity of insulin that the animal is capable of destroying lends emphasis to the hypothesis that the destruction of endogenous insulin may be a major factor in the development of insulin insufficiency (Mirsky, 1952). These observations emphasize also that it is erroneous to consider the regulation of insulin metabolism only in terms of insulin production as has been the practice in the past. Both the secretion and the destruction of a hormone must be evaluated before the mechanism responsible for its insufficiency or excess is established.

Although valuable information concerning the destruction of insulin can be gained from studies with extracts or slices of tissues, such procedures do not yield precise data about the factors which accelerate or inhibit the de-

struction of insulin in the intact organism. The procedure described herein permits the description of such factors.

SUMMARY

Utilizing I^{131} labeled insulin, it has been demonstrated that the intact mouse destroys exogenous insulin at a relatively rapid rate. The reaction appears to follow first order kinetics and is attributed to the action of "insulinase."

REFERENCES

GREELY, P. O.: *Am. J. Physiol.* **129:** 17. 1940.

HAUGAARD, N., M. VAUGHAN, E. S. HAUGAARD, AND W. C. STADIE: *J. Biol. Chem.* **208:** 549. 1954.

LINEWEAVER, H. AND D. BURK: *J. Am. Chem. Soc.* **56:** 658. 1934.

MIRSKY, I. A.: *Rec. Prog. in Hormone Research* **7:** 437. 1952.

MIRSKY, I. A. AND R. H. BROH-KAHN: *Arch. Biochem.* **20:** 1. 1949.

MIRSKY, I. A. AND G. PERISUTTI: *Endocrinology* **52:** 698. 1953.

MIRSKY, I. A., G. PERISUTTI, AND F. J. DIXON: *Proc. Soc. Exp. Biol. and Med.* **86:** 228. 1954.

MIRSKY, I. A., G. PERISUTTI AND F. J. DIXON: *J. Biol. Chem.* (May, 1955).

MIRSKY, I. A., C. J. PODORE, J. WACHMAN AND R. H. BROH-KAHN: *J. Clin. Invest.* **27:** 515. 1948.

TOMIZAWA, H. H., M. L. NUTLEY, H. T. NARAHARA, AND R. H. WILLIAMS: *J. Am. Chem. Soc.* **76:** 3357. 1954.

NOTES AND COMMENTS

IN VITRO SYNTHESIS OF DEHYDROEPIANDROSTERONE SULFATE[1]

De Meio, *et al.* (1952, 1953, 1954) and Bernstein and McGilvery (1952a, b) have shown that rat liver preparations can synthesize the sulfuric acid esters of ordinary phenols. It is well known that several steroids (androsterone, dehydroepiandrosterone, estrone and Δ^{16}-Allopregnene-3β-ol-20-one) are found in human urine as sulfates (Dorfman and Ungar, 1953) and this is evidence for their "in vivo" formation. With the same type of liver preparations used for the study of the formation of sulfuric acid esters of phenols we have been able to demonstrate the "in vitro" formation of dehydroepiandrosterone sulfate. The requirements for this synthesis appear to be the same as those found for the formation of phenyl sulfates (De Meio *et al.*, 1953; Bernstein and McGilvery, 1952a).

EXPERIMENTAL

A 20% homogenate in 0.1M potassium phosphate buffer pH 7 was prepared, with a Potter-Elvehjem type tissue homogenizer, from livers obtained from male albino rats (Albino Farms, Red Bank, New Jersey) immediately after killing. A "microsome-free" solution was obtained from this homogenate with the technique described in a previous paper (De Meio *et al.*, 1953). The "microsome-free" solution was lyophilized and kept at $-20°$. The activity of this preparation in the formation of p-nitrophenyl sulfate has not changed appreciably for a period of a year. The lyophilized material when required for use in experiments was brought to the initial concentration with distilled water. The incubation was carried out in a medium of the following composition: 0.05M potassium phosphate buffer pH 7, 0.005M Mg^{++}, 0.015M $SO_4^{=}$, 0.005M ATP,[2] 0.69×10^{-4}M dehydroepiandrosterone (DHA). The DHA was dissolved in 95% alcohol (30 μg. per 0.1 ml.) and 0.2 ml. of this solution added with continuous shaking to the vessel already containing the enzyme preparation and kept at 0–5°. The preparation, with a final volume of 3 ml., was incubated for 2 hours at 37.5° in air. After incubation 3 volumes of 95% alcohol were added per volume of fluid in the vessel, the protein precipitate separated by centrifugation and washed 3 times with 2 ml. 95% alcohol, the alcohol washings added to the first alcohol supernatant and evaporated to almost dryness. The residue was taken up with 3 ml. of distilled water and extracted 4 times with 3 ml. peroxide-free ethyl ether to obtain the free DHA, washing the combined ether extracts with 2 ml. of distilled water, which was added to the aqueous phase. The bound DHA was obtained from the aqueous phase by extracting 4 times with 3 ml. of butanol. Ether and butanol were evaporated to dryness and the residue taken up in redistilled 95% alcohol and DHA determined colorimetrically applying the Zimmermann reaction according to the technique of Pincus and Pearlman (1941) using the Evelyn photocolorimeter and filter 515. According to Bitman and Cohen (1951) the chromogenic value of DHA sulfate is equivalent to its DHA content. We have confirmed this observation and checked our technique of determination using pure DHA and DHA sulfate. The blank values corresponding to the enzyme preparation were determined and substracted from the values found for the samples. The average blank values for the ether extract corresponded to 6 μg. of DHA and for the butanol extract corresponded to 11.5 μg. of DHA.

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[1] These studies were aided in part by contract Nonr 229 (00) with the Office of Naval Research and by research grant G-3839 from the National Institutes of Health, PHS.

[2] Disodium ATP from the Pabst Laboratories, Milwaukee, Wisconsin.

TABLE 1. SYNTHESIS OF DEHYDROEPIANDROSTERONE SULFATE BY RAT LIVER "MICROSOME-FREE" SOLUTION

Preparations	Bound DHA (in μg.)	Additions
Boiled	0	ATP + Mg^{++} + $SO_4^{=}$
Dialyzed	2.3	—— + Mg^{++} + $SO_4^{=}$
Dialyzed	5.2	ATP + —— + $SO_4^{=}$
Dialyzed	9.4	ATP + Mg^{++} + ——*
Dialyzed	27.8	ATP + Mg^{++} + $SO_4^{=}$

60 μg. of DHA per vessel.
* The equivalent amount of potassium chloride was substituted for the potassium sulfate.

RESULTS

In Table 1 some representative figures clearly indicate that the "microsome-free" solution is able to produce binding of DHA. There is about 66% decrease in the binding of DHA when sulfate is not present and a greater decrease is observed when ATP or Mg^{++} are left out.

DISCUSSION

The fact that a threefold increase in binding of DHA is observed when sulfate is added is indirect evidence for the formation of dehydroepiandrosterone sulfate. Whatever binding takes place when sulfate is not added to the medium may be attributed to another form of binding (possibly glucuronide). The results indicate that there is a requirement for ATP and Mg^{++} and in this respect the system behaves like that involved in the synthesis of sulfuric acid esters of phenols (De Meio *et al.*, 1953; Bernstein and McGilvery, 1952a).

SUMMARY

The *in vitro* synthesis of what appears to be dehydroepiandrosterone sulfate, with rat liver preparations, has been demonstrated. The system requires Mg^{++} and ATP for activity.

Acknowledgments

The authors are indebted to Dr. W. H. Pearlman for advice on the problem of steroid determination.

Dehydroepiandrosterone sulfate was supplied through the kindness of Dr. A. F. St. Andre of Ciba Pharmaceutical Products, Summit, N. J.

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REFERENCES

BERNSTEIN, S. AND R. W. MCGILVERY: *J. Biol. Chem.* **198:** 195. 1952a.
BERNSTEIN, S. AND R. W. MCGILVERY: *J. Biol. Chem.* **199:** 745. 1952b.
BITMAN, J. AND S. L. COHEN: *J. Biol. Chem.* **191:** 351. 1951.
DE MEIO, R. H. AND L. TKACZ: *J. Biol. Chem.* **195:** 175. 1952.
DE MEIO, R. H., M. WIZERKANIUK AND E. FABIANI: *J. Biol. Chem.* **203:** 257. 1953.
DE MEIO, R. H., M. WIZERKANIUK AND I. SCHREIBMAN: *Fed. Proc.* **13:** 198. 1954.
DORFMAN R. I. AND F. UNGAR: Metabolism of steroid hormones (Burgess Publishing Co., Minneapolis) 1953, page 26.
PINCUS, G. AND W. H. PEARLMAN: *Endocrinology* **29:** 413. 1941.

EFFECTS OF ESTROGENS ON BONE DENSITY IN ENGLISH SPARROWS

Estrogenic substances stimulate endosteal growth in birds (Pfeiffer and Gardner, 1938) and mice (Gardner and Pfeiffer, 1938). These effects usually have been evaluated on the basis of proliferation of medullary bone as determined from sections. The following studies, utilizing bone density as an index of growth, were carried out in an attempt to obtain a more objective index of osteotropic action.

Twelve wild-caught (January, 1954) male English Sparrows (*Passer domesticus*) were used in these experiments.[1] They were divided into three groups of 4 birds each. Group I served as controls and received 0.05 ml. corn oil daily; group II received 20 μg. of estrone and group III, 50 μg. of methallenestril (Vallestril) in 0.05 ml. corn oil per day. Intramuscular injections were continued for 19 days. At autopsy, 24 hours after the final injection, the long bones of the left side were removed grossly, autoclaved at 15 pounds pressure for 15 minutes, and cleaned of adherent tissue. The cleaned bones were dried to constant weight at 70° C. and weighed on a Roller-Smith torsion balance.

Densities were obtained by saturating the bones with distilled water to constant weight and weighing them in water and in air to the nearest 0.05 mg. Calculations were based upon the following relationship:

$$\rho_x = \frac{\rho_1}{1 - \frac{W_1}{W_a}}$$

where ρ_x is the density of the bones; ρ_1 the density of distilled water at the temperature at which the weighing was made; W_1 weight of the bone suspended in water, and W_a weight of bone in air. Because it was found that the surface tension of the water exerted a force equal to about 2 mg. on the suspending fibre, a wetting agent, Wetanol, was added to the water to reduce surface tension (20 mg. Wetanol per 100 ml. of water).[2]

Dry weights and densities of bones are presented in Table 1. Although only the femora showed significantly increased densities under stimulation with both estrogens, all mean values for all bones of treated groups were increased over controls. The tibio-tarsus of estrone-treated birds was also significantly more dense, and if one aberrant bone is removed from the calculations the Vallestril-treated group shows a significant increase in density. None of the dry weights showed significant difference between groups.

These results agree well with studies employing radiocalcium (Govaerts, Dellemagne and Melon, 1951) which showed the femur and tibia to be the bones most responsive to estrogenic treatment in pigeons. These data are also of interest in view of the recent implication of hyperestrogenism in the etiology of osteopetrosis (Borellini and Camurati, 1952).

SUMMARY

Estrogens produced significant increase in density of the femur and tibio-tarsus of English sparrows.

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[1] Birds were obtained through the kindness of Dr. Albert Wolfson of Northwestern University.

[2] I am indebted to Mr. H. S. Backus of G. D. Searle & Co. for suggestions on the physical aspects of these problems.

TABLE 1. DRY WEIGHTS AND DENSITIES OF LONG BONES FROM ESTROGEN-TREATED ENGLISH SPARROWS

Group	Treatment	Humerus			Radius			Ulna			Femur			Tibio-Tarsus		
		No.	Dry wt. (mg.)	Density	No.	Dry wt. (mg.)	Density	No.	Dry wt. (mg.)	Density	No.	Dry wt. (mg.)	Density	No.	Dry wt. (mg.)	Density
I	Oil only	4	56.70 ±1.30	1.4098 ±0.0123	4	9.41 ±0.25	1.5246 ±0.0296	4	36.25 ±0.95	1.4806 ±0.0126	4	28.59 ±0.17	1.3122 ±0.0082	3	45.48 ±0.36	1.3456 ±0.0091
II	Estrone 20 μg. per day	3	57.18 ±2.71	1.4419 ±0.0191	3	10.15 ±0.68	1.5807 ±0.0459	3	37.02 ±1.25	1.5124 ±0.0133	3	29.78 ±1.09	1.3622 ±0.0108*	3	45.93 ±1.76	1.3935 ±0.0058*
III	Methallenestril (Vallestril) 50 μg. per day	4	56.26 ±2.24	1.4487 ±0.0104	4	9.06 ±0.59	1.6250 ±0.0431	4	35.91 ±1.52	1.5072 ±0.0129	4	28.93 ±0.69	1.3537 ±0.0119*	4 (3)	43.09 ±1.54	1.4171 ±0.0296 (1.4465 ±0.0041†)

* $P < 0.05$ that treat mean = control mean.
† $P < 0.01$ that treat mean = control mean.

REFERENCES

BORELLINI, A. AND C. CAMURATI: *Minerva Ortopedica* **3**: 1. 1952.
GARDNER, W. V. AND C. A. PFEIFFER: *Proc. Soc. Exp. Biol. & Med.* **37**: 678. 1938.
GOVAERTS, J., M. J. DELLEMAGNE AND J. MELON: *Endocrinology* **48**: 443. 1951.
PFEIFFER, C. A. AND W. V. GARDNER: *Endocrinology* **23**: 485. 1938.

NEW BOOK REGISTER

ENDEMIC GOITER. John B. Stanbury, Gordon L. Brownell, Douglas S. Riggs, Hector Perinetti, Juan Itoiz and Enrique B. De. Castillo. The adaptation of man to iodine deficiency. Cambridge, Massachusetts: Harvard University Press, 1954. 206 pages. $4.00

THE DETERMINATION OF ADRENOCORTICAL STEROIDS AND THEIR METABOLITES. Proceedings of a conference held by the Society for Endocrinology at the Medical Society of London on 21 May, 1953. Memoir No. 2 of the Society for Endocrinology. Edited by P. Eckstein & S. Zuckerman, 33 contributors. London: Dennis Dobson LTD.

BEYOND THE GERM THEORY. Iago Galdston, Editor. 9 contributors. This book derives from the 12th Annual Eastern States Health Education Conference sponsored by the New York Academy of Medicine. It deals with factors other than germs that engender illness and poor health. Number 10 Downing Street, New York 14, N. Y.: Health Education Council, 1954. 170 pages. $4.00

THE PINEAL GLAND, Julian I. Kitay and Mark D. Altschule. A review of the physiologic literature. Cambridge, Massachusetts: Harvard University Press, 1954. 104 pages, 1028 references and 200 Appendices to Bibliography. $5.00

LABORATORY AIDS IN ENDOCRINE DIAGNOSIS. Roberto F. Escamilla. Springfield, Illinois: Charles C Thomas, 1954. 131 pages. $4.75

MEREDITH'S HYGIENE 5th Edition. Arthur F. Davis and Warren H. Southworth. A textbook for college students on physical, mental, and social health from personal and public aspects. New York, N. Y.: The Blakiston Company. 1954. 847 pages $6.00

CONNECTIVE TISSUE IN HEALTH AND DISEASE. Edited by G. Asboe-Hansen, 8 contributors. Copenhagen: Ejnar Munksgaard, 1954. 320 pages. $7.50

THE DIAGNOSIS AND TREATMENT OF THE INFERTILE FEMALE. Fred A. Simmons. Springfield, Illinois: Charles C Thomas, 1954. 73 pages. $2.50

LEUKAEMIA RESEARCH. A Ciba Foundation Symposium. This volume describes the results of an international meeting of noted authorities on the present status of research in leukaemia and the directions in which further research might most profitably be pursued. Boston: Little, Brown and Company, 1954. Illustrated. 290 pages. $6.75

HYPERTENSION. A Ciba Foundation Symposium. This volume discusses the problem of hypertension and its effect on the cardiovascular system from the functional point of view. Boston: Little, Brown and Company, 1954. 73 illustrations. 285 pages. $6.75

THE KIDNEY. A Ciba Foundation Symposium. The ground covered at this symposium included structural and functional relationships in the kidney, the regulation of acid-base balance, general problems of electrolyte excretion, and the renal share in the volume control of body fluid. Boston: Little, Brown and Company, 1954. 125 illustrations. 322 pages. $6.75

PRINCIPLES OF BIOCHEMISTRY. Abraham White, Philip Handler, Emil L. Smith and DeWitt Stetten, Jr. New York 36, N. Y.: McGraw-Hill, 1954. 1117 pages. $15.00

EAT, THINK AND BE SLENDER. Leonid Kotkin. New York 11, N. Y.: Hawthorn Books, Inc. 220 pages. $2.95

COLOR ATLAS OF PATHOLOGY, Vol. II. Prepared under the auspices of the U. S. Naval Medical School of the National Naval Medical Center, Bethesda, Maryland. Philadelphia 5, Pa.: J. B. Lippincott Co. Illustrated with 1032 figures in color on 343 plates. 432 pages. $20.00

HARRISON'S PRINCIPLES OF INTERNAL MEDICINE, Second Edition. 85 contributors. Many of the chapters have been revised extensively and others have been rewritten entirely. New York 22, N. Y.: The Blakiston Company, 1954. 1703 pages. $16.00

REPRODUCTIVE SYSTEM, Volume 2. Prepared by Frank H. Netter. A compilation of paintings on the normal and pathologic anatomy of the reproductive system. Edited by Ernst Oppenheimer with foreword by John Rock. Summit, New Jersey: Ciba Pharmaceutical Products, 1954. 229 full-color paintings. 270 pages. $13.00

ROBERTA COWELL'S STORY. An Autobiography. New York, N. Y.: British Book Centre. 208 pages. $3.00

SYMPOSIUM ON PROBLEMS ON GERONTOLOGY. Proceedings of a Symposium held under the auspices of The Johns Hopkins University, School of Hygiene and Public Health and the National Vitamin Foundation. 10 contributors. New York 22, N. Y.: The National Vitamin Foundation, Inc. 136 pages. $2.50

SEROLOGICAL APPROACHES TO STUDIES OF PROTEIN STRUCTURE AND METABOLISM. Edited by Wiliam H. Cole. Contributors: Alan A. Boyden, Melvin Cohn, David Gitlin, Felix Haurowitz, Michael Heidelberger and J. Munoz. New Brunswick, N. J.: Rutgers University Press, 1954. $2.00

NEWER CONCEPTS OF THE CAUSES AND TREATMENT OF DIABETES MELLITUS. Proceedings of the Symposium on Diabetes sponsored by the New York Diabetes Association and held at Memorial Hospital and The New York Academy of Sciences, New York City. February, 1954. New York 22, N. Y.: The National Vitamin Foundation, Inc. 177 pages. $2.50

DOUBLEDAY PAPERS IN PSYCHOLOGY. Double Day and Company, Inc. Garden City, New York.

The Sexual Nature of Man. Clarence Leuba, Antioch College. 85¢

Feelings and Emotions. Lawrence K. Frank, Formerly director of Caroline Zachry Institute of Human Development. 85¢

Psychology as a Profession. Robert I. Watson, Northwestern University. 95¢

Art and Play Therapy. Emery I. Gondor, New York Medical College and Flower and Fifth Avenue Hospitals. 95¢

The Anatomy of Personality. Donald K. Adams, Duke University. 85¢

On the Nature of Psychotherapy. Arnold Bernstein, Queens College. 85¢

Perception, William H. Ittelson and Hadley Cantril, Princeton University. 85¢

Intelligence. L. J. Bischof, Southern Illinois University. 85¢

Nature and Nurture. John L. Fuller, R. B. Jackson Memorial Laboratory, Bar Harbor, Maine. 85¢

BOOK REVIEW

PRINCIPLES OF BIOCHEMISTRY. 1954. By Abraham White, Ph.D., Philip Handler, Ph.D., Emil L. Smith, Ph.D., and DeWitt Stetten, Jr., M.D., Ph.D. Published by McGraw-Hill Book Company, Inc., New York. pp. 1117, 138 text figures, 87 tables.

The work under review is the latest of a group of new textbooks of biochemistry that have appeared in the last several years. Their novelty lies in emphasis upon the biochemical details of cellular processes, a modern movement initiated in 1947 by Baldwin's lively and graceful "Dynamic Aspects of Biochemistry." The new texts take origin from a shift in the attention of biochemists toward ideas and methods relating to the cell and its parts rather than to the organism and its organs, from the consequent immense accumulation of detailed knowledge of enzymatic processes, and from the necessity of presenting an orderly introduction to the concepts and general principles of cellular metabolism and of relating this new material to past knowledge and thought. Only brief reflection is required to recognize the awesome task set for the authors of a modern textbook of biochemistry. One admires their gallantry and fortitude even while regretting that so often the reach exceeds the grasp.

The first three parts of the present work, comprising rather more than half the text, introduce the fundamentals: the chemistry of cell constituents; catalysis; and metabolism. The last four parts represent special aspects of applied biochemistry or of the development of the subject from particular points of view: body fluids; biochemistry of specialized tissues, biochemistry of the endocrine glands; and nutrition. The ordering of the topics is reasonable, and the treatment is, on the whole, well balanced in the light of the scope and requirements of the whole text. The nine chapters on metabolism make up about thirty per cent of the book. They are comprehensive in respect to the intracellular chemical transformations of the major foodstuffs. The concepts and principles of cellular metabolism are admirably presented and luxuriously illustrated. One chapter, on common pathways in metabolism, makes a praiseworthy attempt to relate the diverse cellular processes to what might one call a "metabolic plan" of the cell. The section on metabolism does not, however, convey any quantitative sense of the larger motions of metabolism, of ebb and flow, and of balance, which characterize the whole organism. For the clinician-physiologist the fundamental principles of chemical transformations in cells are essential to the understanding and interpretation of many of his problems, but an effective grasp of the organism in action cannot be derived in any direct way from those principles. To some extent the last four parts of the book make up for this by their treatment of biochemical events in larger spheres of action. The section on endocrines, which is concise, well-organized and nicely presented, is primarily biochemical in point of view. The opportunity afforded by the endocrine system for presenting an outline of metabolic interrelationships is not taken. Thus in this work, as in each of the other recent textbooks of biochemistry, a picture of the metabolism of the organism does not quite come through. None of them achieves a true integration of fundamentals.

The book as a whole is remarkably even in tone. The individualities of the four authors have been successfully merged in a sober and undemonstrative style. The text is almost free from errors of printing, and the indexing is thoughtful and thorough. Although the book is rather sparsely illustrated there are many reactions and reaction schemes, and these, set off from the text in a clean bold-face, are clear and easy to follow. There are few direct references in the text, but at the end of each chapter, or group of chapters there are references to books, review articles and original papers. These have been judiciously selected and they furnish an excellent guide to further reading.

The authors may be congratulated on their contribution to the new group of working textbooks of biochemistry. Their work can stand respectably with the others, and it may be admired for its many excellences. No student who works with it will fail to acquire a lasting appreciation of the broad scope and fundamental importance of biochemistry.

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THE 1955 ANNUAL MEETING

The Thirty-seventh Annual Meeting of The Endocrine Society will be held in the Chalfonte-Haddon Hall Hotel, Atlantic City, New Jersey, on Thursday, Friday and Saturday, June 2, 3 and 4, 1955.

Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Chalfonte-Haddon Hall Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 3, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately, as the hotels expect to be filled to capacity. Correspond directly with Chalfonte-Haddon Hall, advising time of arrival and departure. Make your reservations now and avoid disappointment.

THE SEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE INDIANA UNIVERSITY SCHOOL OF MEDICINE AT THE CONTINUATION STUDY CENTER OF THE INDIANA UNIVERSITY MEDICAL CENTER, INDIANAPOLIS, INDIANA— SEPTEMBER 26–OCTOBER 1, 1955

The faculty will consist of twenty-one eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies with emphasis on the clinical aspects, with demonstrations of laboratory tests, presentations of cases, and question and answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program, write Post Graduate Office, Indiana University Medical Center, 1100 West Michigan Street, Indianapolis 7, Indiana. Registration is limited to 100; tuition fee is $100.00. Arrangements for housing accommodations will be made through the Indiana Medical Center.

THE LAURENTIAN HORMONE CONFERENCE

The Laurentian Hormone Conference of the AAAS will hold its 1955 annual meeting at the Hotel Stanley, Estes Park, Colorado, during the period September 11–16. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Massachusetts, for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than May 6 in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. Hormone Biosynthesis and Metabolism

The Biosynthesis and Metabolism of Thyroid Hormone.
J. Roche, College de France

The Adrenal Medulla and the Formation of Pressor Amines.
Paul Hagen and A. D. Welch, Yale University Medical School

Biosynthesis of the Sterols and Steroid Hormones, with Particular Reference to the Estrogens.
R. D. H. Heard, McGill University School of Medicine

Biogenesis of Neutral Steroid Hormones.
Mika Hayano, N. Saba, R. I. Dorfman and O. Hechter, The Worcester Foundation for Experimental Biology

Enzymatic Mechanisms of Steroid Metabolism. (a) Oxidation-Reduction Reactions of the Steroid Nucleus. (b) Synthesis of Steroid Glucuronides.
K. J. Isselbacher and G. Tomkins, National Institutes of Health

II. Hormones and Metabolism

Influence of Steroids on Cerebral Metabolism in Man.
G. S. Gordon, University of California Medical Center

Studies of Aldosterone in Relation to Water and Electrolyte Balance.
J. A. Luetscher, Jr., Stanford University School of Medicine

Tissue Metabolism and Growth.
M. E. Krahl The University of Chicago

III. Pituitary Hormones

Human Urinary Gonadotrophin.
A. Albert, Mayo Clinic

Hormonal Control of Melanin Pigmentation.
A. B. Lerner, University of Oregon Medical School

Pituitary Syndromes in Man.
R. F. Escamilla, San Francisco, California

IV. Sex Hormones

Male Sex Hormone and Its Role in Reproduction.
Thaddeus Mann, University of Cambridge, England

Clinical Studies of Testicular Hormone Production.
W. O. Maddock, C. A. Paulsen, and R. B. Leach, Wayne University College of Medicine

The Mechanism of Progesterone Effect on Uterine Muscle.
A. Csapo, Carnegie Institution of Washington